

**Studies on Inter and Intra- Genetic Distance among
Trifolium species based on Morphological,
Biochemical and Cytogenetical Attributes**

THESIS

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**DOCTOR OF PHILOSOPHY
(BOTANY)**

**BY
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2001

4658

DEDICATED TO THE

LOVING MEMORY OF
MY ELDER BROTHER

Dr. S.K.UJALAYAN


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CERTIFICATE

It is certified that this thesis entitled "Studies on inter and intra-genetic distance among *Trifolium* species based on morphological, biochemical and cytogenetical attributes" is an original piece of work done by Shri Bijendra Kumar, M.Sc. (Botany) under my supervision and guidance for the degree of Doctor of Philosophy in Botany, Bundelkhand University, Jhansi.

I, further certify that:

- It embodies the original work of candidate himself
- It is up to the required standard both in respect of its contents and literary presentation for being referred to the examiners.
- The candidate has worked under me for the required period at Indian Grassland and Fodder Research Institute Jhansi.
- The candidate has put in the required attendance in the department during the period (December, 1997 to till date).


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DECLARATION

I hereby declare that the thesis entitled "Studies on inter and intra-genetic distance among *Trifolium* species based on morphological, biochemical and cytogenetical attributes" being submitted for the degree of Doctor of Philosophy in Botany, Bundelkhand University, Jhansi (UP) is an original piece of research work done by me under the supervision of Dr. D. R. Malaviya, IGFRI, Jhansi and to the best of my knowledge, any part or whole of this thesis has not been submitted for a degree or any other qualification of any university or examining body in India / elsewhere.


(BIJENDRA KUMAR)

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3/2/2001
(Bijendra Kumar)

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INTRODUCTION

Introduction

The genus *Trifolium* commonly called clovers, comprises of 237 annual and perennial species (Zohary & Heller, 1984), out of which a few are agriculturally important as cultivated and pasture crops. The important perennial pasture clover *T. repens* (white clover), *T. hybridum* (alsike clover), *T. pratense* (red clover) and *T. ambiguum* (caucasian clover) are widely distributed in the temperate and subtemperate regions of the world. The annual types *T. resupinatum* (persian clover) and *T. alexandrinum* (Egyptian clover or berseem) are commonly cultivated as winter annuals in the tropical and subtropical regions.

Egyptian clover is popularly known as Berseem. The name Berseem is derived from Arabic name 'Bersym' or 'Berzum' (Shukla & Patil, 1985). The crop is believed to be indigenous to Egypt (Narayanan & Dabadghao, 1972) but has domesticated well in India. Berseem is thought to have originated in Asia Minor, from where it was brought to Egypt through Palestine and Syria. It was probably the earliest forage crop grown in Egypt, existing there since the first dynasty in the period 3800-3500 BC. Based on several reports, it is considered that it did not originate in Egypt because authentic seed of this crop has never been discovered in any tomb. However, a Byzantine variety (*Trifolium alexandrinum* var. *Phleoids* Boiss) existed in Kilsali near Smyrna. In India it was introduced into Sindh state (now in Pakistan) in 1904 for the first time (Singh, 1993).

Berseem is one of the true clovers. It is an annual plant (30 to 90 cm) with hollow and very succulent stem. The roots do not extend far into the soil (about 30 cm deep) and are of medium size, being long and tapering, branched, fibrous, and single or clustered. The stems are decumbent, ascending somewhat with prominent transverse rings, diffuse, fistulous, glabrous at the base and increasingly pilose above. The leaves are trifoliate and petiolate and oblong lanceolate to oblong elliptical leaflets, pubescent on both sides. Berseem has an inflorescence of dense head terminating the stem and branches. The seeds are suborbicular to obvoid, 2

mm long. Seed-coats are first dull, becoming shiny on exposure, glabrous, and yellow tinged with brown in the region of hilum and the chalaza.

Berseem (*T. alexandrinum*) is the most important winter season legume cultivated in an area of around two million hectares in India. The significance of this forage species lies in the development of milk industry. It appeared to behave as a most potent milk multiplier in the lactating buffaloes, Sahiwal cows and cross bred cattle as compared to other forage crops alone or in combination (Patil, 1982). Of the two Egyptian biotypes of berseem 'Mescavi' and 'Fahli' introduced in India during 1903, the former proved to be highly adaptable and productive as fodder crop for wide scale cultivation. Most of the present day cultivars are derivatives of Mescavi. The merit of these cultivars lies in their multicut nature (4 - 8 cuts), long duration of green fodder availability (November to April) and very high green fodder yield (85 t/ha), better quality (20% crude protein), high digestibility (up to 65%) and palatability. The phenomenal success of berseem in India is also due to its very high nitrogen fixing ability resulting in substantial improvement in soil fertility.

Considering its high production potential and wide adaptation in the tropical and sub tropical zone of the country, serious attention has been paid for its further genetic improvement. In spite of continuous efforts being made at Indian Grassland and Fodder Research Institute, Jhansi; Punjab Agricultural University, Ludhiana; Jawaharlal Nehru Krishi Vishwavidyalaya Jabalpur, Haryana Agricultural University, Hisar and at many other places, there has not been appreciable genetic improvement in the last two decades. The less quantum of breeding output in this species could possibly be due to some inherent bottlenecks which primarily arise from the method of pollination, seed setting, the maintenance of germplasm (Shukla, 1982) and variability in the self compatibility system (Putiyevsky & Katznelson, 1970; Whyte, 1978). Another bottleneck faced is a narrow genetic base in the crop and efforts for broadening its genetic base have also not been successful. Hence, interspecific hybridization for introduction of alien genes seems to be the possible alternative for enhancing diversity in the

gene pool of *T. alexandrinum*. Long history of inbreeding and selection for high performance genotypes (for yield) has lead to reduction of genetic variability in the genotypes. So far very little work has been done in making interspecific crosses probably because of its failure in natural conditions. To make a serious effort in this direction, it is important to have clear understanding of the affinity among different species of the genus. The phylogeny / affinity of different species of the genus particularly with reference to *T. alexandrinum* is not very clear. Hence, this information needs to be generated, which could be utilized in transferring alien genes into *T. alexandrinum* through conventional / non-conventional breeding approaches.

The major limitations encountered in the present day cultivars are slow establishment / growth up to 45 days resulting in late availability of forage and poor first cut yield; susceptibility to drought, very high and low temperature, salinity, alkalinity and to several diseases and low dry matter in initial cuts. The major genes for above mentioned traits mainly disease resistance and wide scale adaptability to varying soil/climatic conditions are reported to be widely distributed in several wild / allied species. These genes could be incorporated into the present day cultivars of the most commonly grown species in India *Trifolium alexandrinum* provided the affinity with other wild/temperate/sub-temperate species of the genus is clear. Very little work has been done on working out affinity/ relatedness of various species, within the genus *Trifolium*. Although sporadic reports are available about affinity of temperate and subtemperate species, reports on their relationship with *T. alexandrinum* or with other tropical species is lacking. Chen and Gibson (1970 a & b, 1972) indicated affinity among *T. repens*, *T. nigrescens*, *T. occidentale* and *T. uniflorum*. Similarly on the basis of easy crossability under natural conditions, Merker (1988) confirmed close affinity between *T. alpestre* and *T. pratense*. Bobrov (1947) and Whyte (1978) reported that *T. alexandrinum* owes its origin to a few wild species such as *T. salmoneum*, *T. berytheum* and *T. apertum*. Successful recovery of viable hybrids between *T. alexandrinum* and *T. resupinatum* have been reported by Selim *et al.* (1977). Though considerable

efforts have been made in obtaining interspecific hybrids through non-conventional breeding techniques involving mostly the temperate and sub-temperate species (Pandey *et al.*, 1987; Merker, 1988; Yamada and Fukuoka, 1986; Przywara *et al.*, 1989; Sawai *et al.*, 1990, 1995; Ferguson *et al.*, 1990; Repkova *et al.*, 1991; Philips *et al.*, 1992) little work has so far been reported in establishing the affinity between species or towards the estimation of intra and inter-species genetic similarities.

The different approaches to estimate the genetic similarity include morphological, cytogenetical and biochemical markers, in the order of precision. In order to understand the relationship among various species, a number of cytogenetic techniques have been carried out. In fact, any technique alone is insufficient to resolve this problem as in many cases, cross incompatibility and hybrid sterility between species hinder the study of their genomic relationships. Morphological and biochemical studies have been carried out by various authors in several species to determine the genomic relationship (Crawford, 1983). Isozyme variations, coupled with the information rendered through morphological and cytogenetical markers provide a useful clue for the estimation of population structures, genetic distances and phylogenetic relationships (both within and between species).

The earlier reports indicated presence of morphological variations in different species of the genus *Trifolium*. Ryding (1991), Beri & Sohoo (1991) and Martiniello *et al.* (1992) reported diversity in different germplasm lines of *T. burchellianum* and *T. alexandrinum*. Morphological diversity supported with enzymatic variation in *T. medium* has been reported by Bulinska (1992). Similarity patterns/genetic distances based on biochemical survey has been worked out in some species of the genus *Trifolium* by Odoardi & Valdicelli (1993). Intraspecific variations have been studied only in some of the species, including *T. hirtum* (Molina & Jain, 1992), *T. pratense* (Hagima & Moisa, 1992), *T. repens* (Lee *et al.*, 1993) and *T. fragiferum*, *T. montanum*, *T. campestre* (Bulinska, 1994).

In light of the above, the present investigation entitled "Studies on inter and intra genetic distance among *Trifolium* species based on morphological, biochemical and cytogenetical attributes" has been envisaged to work out the genomic relationship of various accessions within and between different species of *Trifolium*.

The study has been undertaken with following objectives:

- 1) To determine phenotypic variations within and between different *Trifolium* species and their biochemical characterization.
- 2) To work out interspecific compatibility relationships and to establish their relative affinity.
- 3) To estimate genetic similarity among various species.

REVIEW

2. REVIEW OF LITERATURE

2.1 Taxonomic Position, Geographical distribution & Classification

The genus *Trifolium* of tribe Trifolieae is agriculturally important genus of the family Leguminosae (Fabaceae). In Hand Book of Legumes of world economic importance, Duke (1981) has included 14 species of *Trifolium*, most of which are forage crops. These include Berseem clover (*T. alexandrinum*), Alsike clover (*T. hybridum*), Crimson clover (*T. incarnatum*), Red clover (*T. pratense*), White clover (*T. repens*), Persian clover (*T. resupinatum*) and Subterranean clover (*T. subterraneum*). A few of the 237 species of this genus have actually been cultivated to date (Zohary & Heller, 1984). The genus has wide range of variability for its habit i.e. annual, biennial and perennial herbs. Leaves are mostly trifoliate, very rarely digitate with 5 to 8 leaflets and leaflets are usually toothed and flowers are in heads or short spike, very rarely solitary. Calyx teeth are equal or unequal, petals persistent or deciduous indehiscent.

In an attempt to classify the genus Zohary & Heller (1984) divided genus *Trifolium* into 8 sections i.e. 'Lotoidea', 'Parameşus', 'Mistyllus', 'Vesicaria', 'Chronosemium', 'Trifolium', 'Tricocephalum', and 'Involucrarium'. According to Zohary (1972) the boundaries between species within a section in many cases are extremely difficult to define and there is in almost all sections a striking grouping of the species into well defined natural clusters that have been used to create series. The delimitation of species within these clusters is often difficult because of the wide range of diversity found in many species which in the majority of cases is caused by primary polymorphism and not by hybridization. The existence of a high level of incompatibility between clover species and the means to overcome such incompatibilities has been widely documented (Keim, 1953; Evans, 1962 a & b; Williams, 1988; Przywara *et al.*, 1989; Ferguson *et al.*, 1990). The largest section 'Lotoidea' comprising of 90 species is considered by Zohary & Heller (1984) as an assemblage of species groups which may have served as source taxa for the evolution of other sections. It is the only section shared by the Eastern and Western

Hemispheres, the others being limited to the Eastern Hemisphere or to the New World alone (Zohary, 1972).

Taxonomic treatments have generally been based on geographical distribution. European species (Ascherson & Graebner, 1908; Coombe, 1968) have thus been classified separately from the North American species (Mc. Dermott, 1910), Oriental species (Hossain, 1961; Zohary, 1970), African species (Gillett, 1952), Russian species (Komarov *et al.*, 1934 -1964) and others. *T. repens* which is a native through out Europe and is now widely distributed throughout the world, has been classified in slightly different ways by different authors, who have placed *T. ambiguum*, *T. angulatum*, *T. glomeratum*, *T. hybridum*, *T. isthimocarpum*, *T. michelianum*, *T. nigrescens*, *T. occidentale*, *T. retusum*, *T. tembense* along with many other species with *T. repens* (Williams, 1987). Most authors agree in placing *T. repens* in section 'Amoria' (formerly 'Euamoria'), which is placed by Hossain (1961) and by Ascherson & Graebner (1908) in subgenus 'Amoria'. Coombe (1968) designates this same group as section 'Lotoidea' of subgenus 'Lotoidea'. *T. glomeratum* and *T. suffocatum* which are included in section 'Amoria' by Ascherson & Graebner (1908) and in section Lotoidea by Coombe (1968), are segregated as section 'Micrantheum' by Hossain (1961) and by Zohary (1970), on account of their sessile flower heads and other floral differences.

The detailed review by Gibelli & Belli (1889-93) as quoted by Williams (1987) has been the basis of subsequent treatments, such as that by Ascherson & Graebner (1908) dealing with the species of Central Europe. Hossain (1961) has given a detailed account of the reproductive structures of the genus, particularly as they affect the protection and dispersal of the seeds, and on this basis recognized eight distinct subgenera, some of which are again divided into sections. Zohary (1970) did not recognize subgenera but classified the genus directly into a number of sections.

2.2. Center of origin

Vavilov (1926, 1951) pointed out that an enormous wealth of diversity of a crop or genus is concentrated in relatively small geographical regions, which he

termed as centre of origin, Centre of diversity or Gene Centres. Harlan (1951) further stressed that even in 'vavilovian' centres the diversity of germplasm is far from uniform and still smaller sub regions can be delineated, which he termed as micro centres.

Center of origin of genus *Trifolium* is not clearly defined. Time and again efforts have been made by different workers to identify the center of origin of genus taking into consideration the criteria such as palynological studies, representation of number of species in certain region, extent of intra-species variation in some particular region *etc.* (Table 2.1). According to Zohary & Heller (1984) the evolution of the genus is closely linked with its phyto-geographical relations. Based on such observation Zohary & Heller (1984) indicated the Mediterranean region with 110 species belonging to 7 sections as one of the main centers of distribution of the genus and also a center of domestication and breeding. Another center of distribution is the Californian region which is considered as primary center of speciation of the genus, although the number of the species in this region is lower. Gillett (1952) also indicated the Mediterranean region as the center of origin due to higher number of species belonging to the genus and also due to other genera of the tribe 'Trifolieae' present in this region. Norris (1956) indicated East Africa as the center of origin of the genus, followed by migration of the African species into the Mediterranean area.

Zohary (1972) suggests that some of the species native to western part of America migrated to Asia and then spread to the Mediterranean area where they created a highly diversified speciation center. He supported this proposal with the fact that species belonging to section 'Lotoidea' have the highest concentration and greatest diversity of forms in North America which could have acted as a source for further differentiation of the Mediterranean species. In addition to this, other species of 'Trifolium' section are not represented from North America instead, five species of subsection 'Lupinaster', considered to be the most primitive group are reported from North America. It is indicative that more survey work is required before precise evolutionary interpretations can be made. One way to gain more information on the evolution of the genus is by a study of molecular phylogenies. According to

Table 2.1 Classification of genus *Trifolium* (as per Coombe, 1968)

S. n.	Sub genus	Section	Species
1	Falcatula (Brot.)		<i>T. ornithopodioides</i> L.
2	Lotoidea Pers.	Lupinaster (Fabr)	<i>T. lupinaster</i> L. <i>T. alpinum</i> L., <i>T. pilczii</i> Adamovic
		Paramesus (C. Presl)	<i>T. strictum</i> L, <i>T. nervulosum</i> Boiss. & Heldr.,
		Lotoidea	<i>T. montanum</i> L., <i>T. balbisianum</i> Ser., <i>T. ambiguum</i> Bieb., <i>T. parnassi</i> Boiss. & Spruner, <i>T. repens</i> L., <i>T. occidentale</i> D.E.Coombe, <i>T. pallescens</i> Schreber, <i>T. thalii</i> Vill., <i>T. hybridum</i> L. <i>T. bivonae</i> Guss., <i>T. isthmocarpum</i> Brot, <i>T. nigrescens</i> Viv., <i>T. michelianum</i> Savi, <i>T. angulatum</i> Waldst & Kit, <i>T. retusum</i> L., <i>T. cernuum</i> Brot, <i>T. glomeratum</i> L., <i>T. suffocatum</i> L.
		Cryptoscladium Celak.	<i>T. uniflorum</i> L
		Mistyllus (C.Presl)	<i>T. spumosum</i> L., <i>T. vesiculosum</i> Savi, <i>T. mutabile</i> Portenschl.
		Vesicastrum Ser.	<i>T. physodes</i> Steven ex bieb., <i>T. rechingeri</i> Rothm., <i>T. fragiferum</i> L., <i>T. resupinatum</i> L., <i>T. tomentosum</i> L.,
		Chronosemium Ser.	<i>T. badium</i> Schreber, <i>T. spadiceum</i> L., <i>T. speciosum</i> Willd., <i>T. boissieri</i> Guss., <i>T. lagrangei</i> Boiss., <i>T. brutilum</i> Ten., <i>T. mesogitanum</i> Boiss., <i>T. aurantiacum</i> Boiss. & Spruner, <i>T. dolopium</i> Heldr. & Hausskn., <i>T. patens</i> Schreber, <i>T. aureum</i> Pollich, <i>T. velenovskyi</i> Vandas, <i>T. campestre</i> Schreber, <i>T. sebastianii</i> Savi, <i>T. dubium</i> Sibth., <i>T. micranthum</i> Viv.
3	Trifolium	Trifolium	<i>T. striatum</i> L., <i>T. arvense</i> L., <i>T. affine</i> C.Presl., <i>T. saxatile</i> All., <i>T. bocconeii</i> Savi, <i>T. tenuifolium</i> Ten., <i>T. trichopterum</i> Pancic., <i>T. phleoides</i> Pourret ex Willd., <i>T. gemellum</i> Pourret ex Willd., <i>T. ligusticum</i> Balbis., <i>T. scabrum</i> L., <i>T. dalmaticum</i> Vis., <i>T. filicaule</i> Boiss., <i>T. stellatum</i> L., <i>T. dasyurum</i> C. Presl, <i>T. incarnatum</i> L., <i>T. pratense</i> L., <i>T. pallidum</i> Waldst. <i>T. diffusum</i> Ehrh., <i>T. noricum</i> Wulfen, <i>T. wettsteinii</i> Dorfler, <i>T. ottonis</i> Spruner ex Boiss., <i>T. lappaceum</i> L., <i>T. congestum</i> Guss., <i>T. barbeyi</i> Gibelli & Belli, <i>T. hirtum</i> All., <i>T. cherleri</i> L., <i>T. medium</i> L., <i>T. heldreichianum</i> Hausskn., <i>T. patulum</i> T. ausch, <i>T. velebiticum</i> Degen, <i>T. pignanii</i> Fauche & Chaub. <i>T. alpestre</i> L., <i>T. rubens</i> L., <i>T. angustifolium</i> L., <i>T. purpureum</i> Loisel., <i>T. desvauxii</i> Boiss. <i>T. smyrnaeum</i> Boiss., <i>T. ochroleucon</i> Hudson, <i>T. pannonicum</i> Jacq., <i>T. canescens</i> Willd., <i>T. alexandrinum</i> L., <i>T. apertum</i> Bobrov, <i>T. echinatum</i> Bieb., <i>T. constantinopolitanum</i> Ser. <i>T. latinum</i> Sebastiani, <i>T. leucanthum</i> Bieb., <i>T. squamosum</i> L., <i>T. squarrosum</i> L., <i>T. obscurum</i> Savi, <i>T. clypeatum</i> L.,
		Trichocephalum Koch	<i>T. subterraneum</i> L., <i>T. globosum</i> L., <i>T. pauciflorum</i> D'Urv.,

Doyle (1987) the molecular systematics of legumes will see the extension of these approaches to taxa other than the handful of cultivated legumes that have so far been investigated.

2.3. Geographical distribution of different species

The only species of subgenus 'Falcatula', *T. ornithopodioides* is found in open moist or wet habitat in West Europe northwards to Ireland and Netherlands and extending eastward to Italy and south east part of Europe. The species of subgenus 'Lotoidea' have been reported from Europe (south France, north and south Spain, Britain) the Mediterranean region, southern part of erstwhile USSR, Greece, Romania, Portugal but the majority of species are from Europe. The various species of subgenus 'Trifolium' are reported from Europe, Portugal, Balkan peninsula, Greece and other parts of Mediterranean region (Table 2.2).

Zohary (1972) has mentioned that species of only one section of the genus *i.e.* 'Lotoidea' is represented both in eastern and western Hemisphere or to the new world alone. The boundaries between species in many cases are poorly defined because of wide range of intraspecies diversity which is caused by primary polymorphism and not hybridization. Zohary (1972) has divided the genus into following eight section :

1: Lotoidea – The largest section of 90 species is distributed across the North Hemisphere from E to W pacific. This is considered to be the oldest section as it harbors most ancestral forms of the genus. Its wide distribution suggests its existence during the period when two hemispheres were easily transversable *i.e.* in Neogene. On the assumption that this section served other sections which were derived from certain parts of this section, the long span of time needed for this evolutionary process is quite comprehensible. The main traits of this section are bracteate and pedicellate flowers, regular calyx with its open or almost open throat, 2-4 (-8) seeded legume. The pod as well as other characters resemble typical leguminous pattern conforming to its primitive nature.

2: Trifolium- It comprises of 73 species which are heterogeneous in appearance and polymorphic for many characters. Sessile ebracteate flower, throat of calyx tube

Table 2.2. Origin, Domestication , habit and somatic chromosome number of different *Trifolium* species

S.N.	Species	Habit	Diploid number	Origin	Domestication
1	<i>T. apertum</i>	Annual	16		S.E.Russia, RS (E), N.W. Caucasus, N.E. Anatalia
2	<i>T. resupinatum</i>	Annual	16	S. Asia Minor & Iran, Mediterranean Greece & Egypt	W Pakistan, Punjab, England, USA, Temperate countries.
3	<i>T. glomeratum</i>	Annual	16,14		SW Europe, England
4	<i>T. subterraneum</i>	Annual		Mediterranean, Europe, Asia, Africa and S England	Australia, New Zealand, US (Coastal areas)
5	<i>T. vesiculosum</i>	Annual	16	W & E Mediterranean, Balkans Peninsular, Greece, Crimea, W Caucasus, S Russia,	S USA, Georgia, Albania & Mississippi to Texas, N to Arkansas, S Carolina and Tennessee
6	<i>T. hybridum</i>	Perennial	16,32	N Europe	Europe/ Central Asia Minor/ Temperate regions
7	<i>T. repens</i>	Perennial	16,32	E. Mediterranean of Asia Minor	
8	<i>T. pratense</i>	Annual/ Biennial	14,28	SW Europe & Asia Minor	N Atlantic & Central Europe, Mediterranean, Balkans, Iran, India, Himalayas, Russia from Arctic south to eastern Siberia, Caucasus.
9	<i>T. incarnatum</i>	Annual	14, 16	Atlantic & S Europe, Caucasus and Trans Caucasus	Southern USA
10	<i>T. hirtum</i>	Annual	10, 16	W & E Mediterranean, Balkans, Asia minor, Trimeia, Caucasus & Trans Caucasus	California

Contd..

Table 2.2. Contd.

S.N.	Species	Habit	Diploid number	Origin	Domestication
11	<i>T. diffusum</i>	Annual/ Biennial	16		S, SE and Central Europe
12	<i>T. spumosum</i>	Annual			
13	<i>T. lappaceum</i>	Annual	16		S. Br S. Europe
14	<i>T. argutum</i>	Annual	16		
15	<i>T. arvense</i>	Annual	14		
16	<i>T. campestre</i>	Annual	14		Through out Europe
17	<i>T. constantinopolitanum</i>	Annual	16		Bulgaria, N Greece, Turkey-in -Europe
18	<i>T. alexandrinum</i>	Annual	16	Mediterranean region Near East, India	Tropical and Sub-tropical regions, California & S. United States of Florida.
19	<i>T. cherleri</i>	Annual	10		
20	<i>T. nigrescens</i>	Annual	16,32	Turkey	US (Alabama) and South states.
21	<i>T. echinatum</i>	Annual			Balkan Peninsular, Italy and S Romania
22	<i>T. medium</i>	Perennial	70-82	Eurasia grassy places	E. Canada & N United states.
23	<i>T. alpestre</i>	Annual	16		C, E & S Europe northwards to Denmark and Estonia
24	<i>T. temense</i>	Annual	16		
25	<i>T. purpureum</i>	Annual	14		
26	<i>T. angustifolium</i>	Annual	14,16		S. Br, S. Europe
27	<i>T. retusum</i>	Annual	16		Dry grassy places, C & SE Europe, NW Spain and E Portugal

provided with hairy or callous ring and single seeded pod are some of distinguishing characters.

3: Vesicaria- This section with 7 species is marked for morphological and ontogenetical development of calyx as a vesicular body serving seed dispersal most efficiently. A few species of 'Lotoidea' resemble this character which may be considered as starting point towards the section of 'Vesicaria'.

4: Mistyllus- It comprises of 9 species having unique structure of calyx and corolla. The section shows the close relationship with 'Lotoidea', in some common floral features like brectate flower, regular calyx limb, 2-4 seeded pod dehiscing suturally. The section is also unique in distribution *i.e.* 6 species in Mediterranean and 3 in tropical Eritreo-Arabian subregion.

5: Paramesus- The structure of pod and occurrence of gland bearing teeth on stipule and the calyx separate this section of two species from 'Lotoidea'.

6: Involucrarium- This is an exclusively American section distinguished on the basis of fissure of the calyx teeth, the sharply dentate or insided stipule and presence of an involucre at the base of head. It is difficult to trace links of this section with 'Lotoidea', but there could be some point of junction.

7: Trichocephalum- The section with 8 species is most natural group, sharply isolated from all other sections as only a small number of flowers of the head are fertile. All others have been converted to a mass of hairs or bristles which help in dispersal. The section is considered to be most advanced because of animochoric, zodochoric or Geocarpic mode of seed dispersal.

8: Chronosemium- Consisting of 21 species and unique floral structure and fruiting calyces. Bilipped calyx, persistent corolla with its spoon or boat shaped standard helping in animochorous dispersal, stipulate, one seeded pod are some of the distinguishing features. The section is also considered to be the advanced one.

2.4. Interspecific crossability

Interspecific hybridization studies have been carried out by various workers involving mainly the temperate species to get the basic information on the phylogenetic relationships. Presence of both pre and post fertilization barriers have been reported in the genus *Trifolium*. The pre fertilization barrier includes failure in pollen germination, abnormal growth of pollen tubes, failure of fertilization whereas post fertilization barriers are mostly marked by embryo abortion (Evans, 1962a & b; White & Williams, 1976; Williams & White, 1976).

Report on successful development of interspecific hybrid first came in 1953 when an octoploid *T. repens* was crossed with tetraploid *T. nigrescens* (Brewbaker & Keim, 1953). Since then several interspecific crosses have been reported by various workers mostly involving *T. repens* as one of the parents (Table 2.3). These hybridization studies have established crossability of *T. repens* with *T. nigrescens*, *T. uniflorum*, *T. isthmocarpum*, *T. ambiguum*, *T. alexandrinum*, *T. semipilosum* and *T. occidentale*. Interspecific hybridization programme involving *T. pratense* has revealed its crossability with *T. demissum* and *T. sarosiense* only (Table 2.3). Numerous attempts at crossing perennial species with *T. pratense* have not been successful (Wexelsen, 1928; Evans & Denward, 1955; Taylor *et al.*, 1963; Kazimierski *et al.*, 1972). However, lack of success in these studies could be attributed to the fact that the crosses involved different taxonomic sections, ploidy levels, and breeding systems.

Some reports of interspecific hybridization using *T. alexandrinum* as one of the parents are available from Israel. Hybridization among species related to *T. alexandrinum* was studied by placing single plants of *T. alexandrinum*, in pots within natural stands of various related species (Katznelson, 1971), and on the basis of results obtained five interspecific crossability groups were recognized. One of these crossability groups included *T. berytheum*, *T. salmoneum*, *T. apertum*, *T. meironense* and *T. alexandrinum*. From these studies it was concluded that *T. berytheum* and *T. salmoneum* and especially *T. salmoneum*, seems to be the progenitor of the cultivated *T. alexandrinum*. The opinion of Bobrov (1947) that *T. apertum* is the wild progenitor of *T. alexandrinum* has not been accepted by

Table 2.3. Interspecific hybridization in *Trifolium*

S. N.	Cross combination	Result	Reference
	By hand pollination		
1	<i>T. repens</i> (8x) x <i>T. nigrescens</i> (4x)	Hybrid developed	Brewbaker & Keim, 1953
2	<i>T. repens</i> x <i>T. uniflorum</i>	Hybrid developed	Pandey, 1957
3	<i>T. pratense</i> x <i>T. demissum</i> .	Hybrid developed	Taylor, 1959
4	<i>T. repens</i> x <i>T. isthimocarpum</i>	Sterile hybrid developed	Kazimierski & Kazimievska, 1972
5	<i>T. alpestre</i> x <i>T. heldreichium</i>	Hybrid developed	Quesenberry & Taylor, 1976
6	<i>T. heldreichium</i> x <i>T. alpestre</i>	Hybrid developed	Quesenberry & Taylor, 1976
7	<i>T. alpestre</i> x <i>T. rubens</i>	Hybrid developed	Quesenberry & Taylor, 1976
8	<i>T. rubens</i> x <i>T. noricum</i>	Hybrid developed	Quesenberry & Taylor, 1976
9	<i>T. ambiguum</i> x <i>T. repens</i>	Fertile hybrids developed after back crossing	Hussain & William, 1997
10	<i>T. sarosiense</i> x <i>T. alpestre</i> (4x)	Hybrid developed	Quesenberry & Taylor, 1978
11	<i>T. alpestre</i> (4x) x <i>T. sarosiense</i>	Hybrid developed	Quesenberry & Taylor, 1978
12	<i>T. repens</i> x <i>T. alexandrinum</i>	F1 seedling died after 2 weeks	Trimble & Hovin, 1960
13	<i>T. repens</i> x <i>T. alexandrinum</i> (both 2x and 4x)	Embryo developed up to 12 days	Evans, 1962a
14	<i>T. repens</i> x <i>T. alexandrinum</i>	Hybrid developed	Selim <i>et al.</i> , 1977
15	<i>T. semipilosum</i> x <i>T. repens</i>	Embryo developed up to 10 to 12 days	White & Williams, 1976
	Using Embryo/ovule culture		
1	<i>T. repens</i> x <i>T. ambiguum</i>	Sterile hybrids developed	Williams, 1978; Anderson <i>et al.</i> , 1991; Yamada <i>et al.</i> , 1989
2	<i>T. repens</i> x <i>T. ambiguum</i>	Fertility of F1 restored by back crossing	Williams & Verry, 1981; Anderson <i>et al.</i> 1991.
3	<i>T. ambiguum</i> x <i>T. repens</i>	Male sterile hybrid developed, fertility restored after back crossing	Meredith <i>et al.</i> , 1995
4	<i>T. sarosiense</i> (2n=48) x <i>T. pratense</i> (2x)	Hybrid developed	Collins <i>et al.</i> , 1981 Phillips <i>et al.</i> , 1982;
5	[{(<i>T. repens</i> x <i>T. uniflorum</i>) x <i>T. occidentale</i> } x <i>T. occidentale</i>] x <i>T. ambiguum</i>	Trispecific hybrid crossed with fourth species	Fergusun <i>et al.</i> , 1990

(Putiyevsky *et al.*, 1975). In an other interspecific hybridization programme recovery of viable hybrids between *T. alexandrinum* and *T. resupinatum* has been achieved by Selim *et al.*(1977) in Egypt.

Embryo culture have been used as effective tool for interspecific hybrids in *Trifolium* (Table 2.3). Some of the novel interspecific combination such as *T. ambiguum* with *T. montanum* and *T. occidentale*; *T. isthimocarpum* with *T. repens* and *T. nigrescens*; a trihybrid of *T. repens* x *T. uniflorum* x *T. occidentale* with *T. ambiguum* was produced in several genotypic combination by using embryo culture technique (Ferguson *et al.*, 1990)

2.5. Cytology

Information on chromosome complements of *Trifolium* is also scanty. Although, the diploid chromosome of large number of species have been reported but detailed study on karyotype and their evolutionary trend and meiotic behavior is not available. The somatic chromosome number of different species has been summarized in Table 2.2. Prichard (1969) reported that most of the species have a chromosome number of $2n=2x=16$; some of them are $2n=10$, 12, 14 or polyploid.

Karpechenko (1925) was first to report first regarding chromosome number of $2n=32$ in *T. repens*. Out of a total of 155 species reported so far, a total of 7 species of *Trifolium* possesses $x=6$ or $x=5$. Pritchard (1969) reported that 62 species of subgenus 'Amoria' possess $n=8$. Polyploidy is uncommon in *Trifolium* and only 16% species are polyploid. However, 70% of known polyploids are from 'Amoria'. In the genus 84% of species possessed $x=8$, 12% $x=7$, and 4% $x=6$ or 5. 'Involucrarium', 'Amoria', 'Mistyllus' and 'Galearia' sections possessed $x=8$ only. The polyploids are frequently indigenous to regions which are far removed from the Mediterranean (considered to be the main center of origin of the genus).

Chromosomes in *Trifolium* species range in length from $1\mu\text{m}$ to about $5\mu\text{m}$. Karyotypic comparison have been carried out by Wexelsen (1928), Pritchard (1962) and Chen & Gibson 1971(a). Two satellite bearing chromosome have been identified in *T. repens* by Wexelsen (1928). Karyotypic similarity of *T. repens* with

T. nigrescens, *T. occidentale*, *T. petrisavii* has been reported (Chen & Gibson, 1971).

In contrast to earlier reports (Karpechenko, 1925; Wexelsen, 1928; Wipf, 1939 and Larsen, 1960) Pritchard (1969) reported 16 chromosomes both in *T. glomeratum* and *T. angustifolium*.

Kumari & Bir (1990) in a review of karyomorphological evaluation in Papilionaceae have reported presence of secondary constriction in submetacentric chromosome of *T. alexandrinum*, the total haploid chromatin length in *T. alexandrinum* (19.82 μm), *T. hybridum* (19.84 μm), *T. resupinatum* (11.59 μm), *T. minus* (23.25 μm) and *T. repens* (47.03 μm). On the basis of Symmetry Index values the karyotype of *T. minus*, *T. repens*, *T. resupinatum* have been considered highly symmetrical whereas that of *T. hybridum* to be moderately symmetrical.

Anderson *et al.* (1972) have studied somatic chromosome number of 17 species of *Trifolium* and found that the arm ratio and relative length of each of chromosome of *T. hirtum* varied from 1.17 to 1.53 and 1.29 to 1.58 in *T. desvauxii*, and possessed $n=5$ chromosomes.

In Ladino clover, Chen and Gibson (1971) identified 4 metacentric or near metacentric chromosome pairs, 11 submetacentric pairs and 1 subtelocentric pair bearing satellites. Close similarities among the karyotype of *T. repens*, *T. nigrescens* and *T. occidentale* indicates close phylogenetic relationships among these species. Such observations are supported by studies of chromosome pairing in interspecific hybrids also.

The majority of *Trifolium* species possess 16 chromosomes ($n=8$) or 14 chromosomes[$(n=7)$ (Pritchard, 1969, Putirevsky & Katznelson, 1970; Chen & Gibson, 1971; Anderson *et al.*, 1972)]. Seven species have been reported to have less than 14 chromosomes as reported by various authors in *T. hirtum* ($2n=10$, Britten, 1963), *T. scabrum* ($2n=10$, Larson, 1960; Kliphus, 1962), *T. cherleri* ($2n=10$, Pritchard, 1967), *T. ligusticum* ($2n=12$, Pritchard, 1967), few genotypes of *T.*

subterraneum (2n=12, Yates and Britten, 1952; Brock, 1953), *T. bocconeii* (2n=12, Anderson *et al.*, 1972), *T. desvauxii* (2n=10, Anderson *et al.*, 1972).

2.6. Biochemical Studies

Reports on biochemical studies in genus *Trifolium* are rare and few studies have been reported on use of isozyme, protein banding and RAPD marker. Collins *et al.* (1984) used isozyme electrophoresis for classification of *T. subterraneum* into 3 subspecies. Similarly, Rudnicka (1975) used serological analysis for species relationship in the genus and found considerable differences in the protein spectra of the species within the same section. Jorgensen and Cluster (1988) have used nuclear ribosomal DNA of some *Trifolium* species to postulate phylogenetic relationship.

In a detailed review Bullitta & Hayward (1996) reported that the chloroplast DNA variation in *Trifolium* show the presence of large dispersal repeats contributing to an unstable and actively rearranged chloroplast genome in *Trifolium* that make the genome of these species different by eight or more inversions from those of other genera of same tribe. Phylogenetic analysis of white clover and related species by RAPD markers revealed similarity among *T. repens*, *T. occidentale*, and *T. nigrescens* (Bullitta & Hayward, 1996). In this study clustering of accessions of *T. occidentale* with *T. repens* and *T. nigrescens* indicated the possible hybridity of these three species.

Abdel-Tawab *et al.* (1985) used isozyme genetic markers as a reliable biochemical marker to predict the yield potential of berseem clover. In his study involving 69 accessions of berseem clover marked differences existed between accessions. Differences in seed protein electrophoretic pattern revealed remarkable correlation with yielding ability of these accessions. Electrophoretic pattern for seed protein revealed 2 to 7 bands. Peroxidase and esterase enzyme were also found to be good markers for identification and discrimination between good and inferior yielders. The good yielder exhibited invariably higher number of bands than poor one. The difference between poor and good yielder lines for peroxidase and esterase enzyme was mainly for the intensity of the bands i.e. low yielder exhibited relatively faint bands. In general these accessions exhibited

presence of only 2 peroxidase bands in PAGE analysis. The esterase zymogram showed the maximum of 7 bands.

Based on SDS electrophoresis for seed protein profile among *Trifolium* species, Abdel-Tawab^{et al.} (1987) found variable relationships between the species. 'Miskawi' and *T. subterraneum* gave a unique profile, while 'Fahli', *T. lappaceum* and *T. hirtum* were partially similar to each other. Considerable resemblance was noted by SDS electrophoresis between *T. alexandrinum*, *T. pratense* and *T. medium*, between *T. resupinatum*, *T. hybridum* and *T. repens*, and between *T. nigrescens* and *T. hybridum*.

Michaelson-Yeates *et al.* (1997), used isozyme markers to determine pollen flow and seed paternity mediated by *Apis mellifera* and *Bombus* species in self-incompatible *Trifolium repens*. Five banding patterns were identified for PGI (Phospho gluco isomerase), a dimeric enzyme for PG1-2 locus were identified in population of *T. repens* cv. S100. In total the two isozyme loci PGI (1) (monomorphic) and PGI (2) (polymorphic) for 5 alleles have been identified (Michaelson-Yeates, 1986)

Page *et al.* (1997) used RAPD analysis for identifying DNA markers linked to *Sclerotinia* crown and stem rot resistance of red clover by bulked segregant analysis. Four RAPD fragments were identified as candidate markers for *Sclerotinia* crown and stem rot resistance of which three are for resistance and one for susceptibility.

2.7. Evolution of Genus

Wexelsen (1928) suggested that hybridization played a minor role in evolution of *Trifolium* because hybrids were rarely found in the nature. It is possible that progenitor species of higher chromosome number will show a close relationship to the lower chromosome number as Pritchard (1969) found evolutionary trend in *Trifolium* towards a reduction in basic chromosome number. If this assumption is valid, the hybrid of species with close but different chromosome number may have a greater chance for success than *Trifolium* species with the same chromosome numbers. The interspecific hybrid obtained between *T. pratense*

($2n=14$) and *T. diffusum* ($2n=16$) by Taylor *et al.* (1963) and also the close relationship between *T. subterraneum* ($2n=16$) and *T. israeliticum* ($2n=12$) reported by Zohary & Katznelson (1958) may illustrate this point.

If white clover is of hybrid origin it could have arisen from a cross between two closely related although divergent populations, perhaps ecotypes, of a single widespread diploid species (Navalikhina, 1977). *T. occidentale* and *T. nigrescens* may present two such ecotypes which have diverged further because of geographic isolation but have nevertheless retained some affinities. On the basis of ease of crossing with white clover, similar karyotypes and chromosome pairing and presence of cyanogenic glucosides, these diploid species are closely related to white clover. *T. ambiguum* is another such closely related species which has evolved into a polyploid series.

Evans (1962a) has suggested that *T. uniflorum* may be an ancestor of *T. repens*. This species is a tetraploid sufficiently different morphologically to be classified into section 'Cryptosciadium' subgenus 'Amoria' and has a distinct karyotype with four satellites (Gibson & Chen, 1971). It forms mainly quadrivalents and bivalents at meiosis and is likely to have had an autotetraploid origin. It contains a trace of cyanogenic glucoside (Gibson *et al.*, 1972). This evidence suggests that *T. uniflorum* arose from a diploid ancestor closely related to the ancestor(s) of *T. repens*. Its possible role in the origin of white clover is unknown.

T. isthmocarpum and *T. argutum* (*T. xerocephalum*) have also been suggested as possible diploid ancestor of *T. repens* on the basis of their crossability with latter species (Kazimierski & Kazimierska 1968, 1972). Both species were crossed with difficulty to *T. repens* but neither gave fertile hybrids. No conclusions can be drawn on the possible significance of these species in the ancestry of *T. repens* until much more research with them is carried out on interspecific hybridization.

Duke (1981) has mentioned in the 'Hand book of Legumes of world' about possible progenitor of Berseem. *T. berytheum* Boiss. & Bl. centered in Israel, Lebanon, and Syria, is fully fertile and is interconnected with berseem through a

series of intermediate forms. It may represent the wild progenitor of berseem. Other species inter fertile with berseem include *T. apertum* Bobr., *T. meironensis* Zoh. & Lern. and *T. salmoneum* Mout assigned to the Mediterranean center of diversity.

2.8. Isozymes in plant biology

Biochemical methods have been widely used for fast identification of cultivars and detection of the inheritance of the multiple forms of a single protein e.g. isozymes. Since their discovery by Hunter & Markert (1957) isozymes have played a key role in many branches of biology. To date, they have become the most widely recognized links between the organismal and molecular approach to our science. Isozymes were originally defined by Markert & Moller (1959) as different variants on the same enzyme, having identical or similar functions and present in the same individual. As such their importance for understanding gene action in development and differentiation was explained during the 1960s in both animals and plants. A review of this early work was made by Scandalios (1969) of six different kinds of enzymes. For some of them he noted difference in different parts of the same plant; both presence vs. absence and quantitative differences in concentration.

The detection of isozymes of a given enzyme depends upon plant age, cell or tissue origin, growth environment and enzyme stability as well as method of extraction, separation and visualization (Sheen, 1983). Most earlier studies employed disc gel electrophoresis and reported isozymes as migrating towards the anode. The use of starch or polyacrylamide gel slab enabled the detection of both anodic and cathodic isozymes. However, considerable variation in the number of isozymes of a given enzyme have been reported because of varying experimental conditions.

Stegemann & Loesch^ecke (1962) reported that the electrophoretic patterns of potatoes were mirror of the cultivars. Enzyme electrophoresis has been shown to be a dependable tool in the identification of cultivars of different plant species (Bassiri & Rouhani, 1977; Gorman & Kaing, 1978; Quiros, 1980). Larsen (1969) claimed that plant breeder can use isozyme systems without any need to make a special effort to attach a unique morphological markers to lines or cultivars.

Polymorphism in cultivars of barley and natural populations of *Avena* were reported by R. W. Allard and his associates (Kahler & Allard, 1970, Marshall & Allard, 1970 a & b). Gottlieb (1971) while reviewing both plant and animal populations have pointed out the inestimable value of isozymes for gaining knowledge about processes of evolution.

For population studies, isozymes (or allozymes, as they are sometime called) make possible comparisons between individuals and populations on the basis of several gene loci, rather than just one or two. Moreover, if the analysis is accompanied by investigation of progenies of the organisms analyzed, Mendelian segregation ratios can be obtained without the trouble of isolating parents and making crosses. Two parameters have been extensively used: the proportion of enzyme loci for which the population is polymorphic [P], and the mean number of loci for which individuals are heterozygous[H].

Isozymes also provide valuable information with respect to hybridization and gene duplication, including polyploidy. With respect to hybrid origin of diploid species, one enzyme comparison (in *Stephanomeria diegensis*) confirmed a hybrid origin hypothesis based upon morphology.

The use of isozymes as markers has been a new approach of considerable value. Doebley (1990) has shown that it can aid greatly our understanding of crop plant evolution. The careful research of Stuber (1990) has used the method for attacking one of the most important and difficult problem in plant breeding, the location, number and nature of the genes that contribute to patterns of quantitative inheritance. These results should improve greatly the efforts to increase grain yield. Torres (1990) has shown that marker isozymes provide valuable short cuts.

Biochemical technique like starch gel electrophoresis, provides additional single gene markers which helps to study evolutionary processes. Isozyme loci have several advantages over single gene morphological traits:

1. genetic inheritance of electrophoretically detectable traits can be easily demonstrated, most loci have discrete Mendelian inheritance,
2. most are co-dominant and allele frequencies can be calculated directly,

3. estimates of levels and distribution of genetic variation can be compared directly between population or species,
4. an array of enzymatic loci can be assayed using small quantities of material, usually one leaf or a seed will suffice,
5. many loci express at all stages of the life cycle,
6. isozymes can be resolved for most plant species regardless of habitat, size or longevity.

Isozyme analysis have been used to describe levels and distribution of genetic variation. Isozyme loci are used as genetic markers to study the evolutionary mechanism that produced genetic structuring in populations. Isozyme have most often been used to estimate levels of variation within population. The most commonly used measures of intra population variation are the percent of polymorphic loci, the number of alleles per locus, the effective number of alleles per locus and the mean proportion of heterozygous loci per individual. The last parameter is the expected mean heterozygosity assuming Hardy- Weinberg equilibrium.

Hamrick *et al.* (1979) noted considerable heterozygosity among species for levels of within population variation. A significant proportion of this variation was associated with life history and ecological characteristics of the species. Species that were widespread long lived, primarily out crossed by wind pollination, had high lifetime fecundates and were characteristic of the later stages of succession maintained higher levels of intrapopulation variation than species with other combinations of traits.

The distribution of allozyme variation among populations is the product of interactions among several evolutionary factors. Of primary importance are selection, effective population size, and the ability of the species to disperse pollen and seeds. In general, selection should increase population differentiation as would genetic drift. Species with more pollen or seed movement should have less differentiation than species with restricted gene flow. In support of these predications, Loveless & Hamrick (1984) found that long lived polycarps common to the later stages of succession had low G_{st} value.

The most convincing study specifically designed to compare variation between allozyme and morphometric traits is that of Price *et al.* (1984). Comparisons were made between estimates of inter populational differentiation based on allozyme polymorphisms and measurement characters in three predominantly self pollinated species, *Avena barbata*, *Hordeum vulgare* and *Hordeum jubatum* and an out crossing species, *Clarkia williamsonii*. For each species, genetic diversity was measured at several enzyme loci and for several morphological traits. Morphometric differences among populations was measured by Mahalanobis distance function and Hedrick's (1971) measure of genotypic distance was used to estimate allozyme variation among populations. The rank correlation between the two distance measures for *Avena barbata* was positive and highly significant ($r=1.00$; $P<0.001$) whereas values for *Hordeum jubatum* ($r=0.47$; $P=0.14$) and *Hordeum vulgare* ($r=0.60$; $P=0.07$) approached significance. The rank correlation for *C. williamsonii* ($r=0.07$; $P=0.64$) was not significant, suggesting that isozyme loci may provide more information about other genes in self pollinated than out crossed plants.

In a broad sense, the term isozyme (also called isoenzyme) refers to any two distinguishable proteins that catalyze the same biochemical reaction. The entire gamut of biochemical techniques, from chromatography to aminoacid sequencing, has been used to distinguish different isozymes. The most common method in use by plant geneticists and breeders is horizontal starch gel electrophoresis, which separates proteins primarily on the basis of charge and size. Indeed, the term isozyme was first used to describe the relation ship between such electrophoretic variants (Hunter & Markert 1957).

Isozyme polymorphism have provided population geneticists and systematists with the simple genetic markers necessary to analyze gene flow, differential selection pressure and genetic relationships among populations and taxa (Gottlieb, 1981). It has been repeatedly demonstrated that genetic diversity is correlated with genetic distance as measured by allozyme variation (Brown & Weir, 1983). Many genera, including important crops, have been subjected to evolutionary and systematic analyses involving isozyme surveys, such as *Zea* (Doebley *et al.*,

1986 ; Kahler *et al.*, 1986; Smith *et al.*, 1985), *Solanum* (Oliver & Martinez-Zapater, 1984), *Cucumis* (Kato *et al.*, 1978; Esquinas, 1981; Perl-Treves *et al.*, 1985), and *Pinus* (Dancik & Yeh, 1983; Loukas *et al.*, 1983; Furnier & Adams, 1986) have been published. In addition, many new genera have been examined, including *Allium* (Hadacova *et al.*, 1981, 1983), *Amaranthus* (Hauptli & Jain, 1984), *Beta* (Shevtsov *et al.*, 1985; Van Geyt & Smed, 1984), *Lactuca* (Kesseli & Michelmores, 1986), *Lens* (Hoffman *et al.*, 1986; Pinkas *et al.*, 1985), *Pisum* (Zimniak-Przybylska *et al.*, 1985), *Plantago* (Van Dijk, 1984), *Populus* (Cheliak & Dancik, 1982; Cheliak & Pitel, 1984), *Pyrus* (Menendez & Daley, 1986), *Setaria* (Kawase & Sakamoto, 1984), and *Vicia* (Yamamoto & Plitmann, 1980).

Yndgaard & Hoskuldsson (1985) identified six uses of isozyme markers in plant germplasm collections (1) description of a population or cultivars, (2) detection of genetic differences among individuals or cultivars, (3) determination of phylogenetic relationships within a species, (4) analysis of migration patterns of a species from centers of origin, (5) identification of duplicate accessions, and (6) aid in the planning of new collection expeditions.

2.9. Different isozyme systems

Acid phosphatase (ACP): Acid phosphatase function in the hydrolysis of phosphomonoesters is important in the variety of biochemical reactions including the formation of sucrose in photosynthesis. The existence of several distinct acid phosphatase (ACPH) enzymes in wheat was suggested over 25 years ago (Roberts, 1956) on the basis of a study of a large number of inhibitors and substrates. As many as nine ACPH isozymes have since been detected in hexaploid wheat using electrophoretic (Hart, 1973) and isoelectric focusing (Akiyama *et al.*, 1981) techniques. Designations of the loci associated with ACP isozymes have been varied including Ap (El-Metainy & Omar, 1981), Acph (Stuber *et al.*, 1980, 1982), Phos (Brown & Allard, 1969).

Study of rye acid phosphatase isozymes were first published by Jaaska (1972, 1975). Morawieca *et al.* (1976) found 4 to 5 isozymes of acid phosphatase in

the rye cultivars and Griffin (1976) has also studied individual variation of isophosphatases in rye.

Esterase: Esterase isozymes have been subject of numerous studies of polymorphisms in maize. The esterase include a host of ester hydrolases; however, the low substrate specificity of maize esterase make a clear distinction among types very difficult. Esterase zymograms are rather complex in rye, being manifested by a series of bands controlled by multiple gene loci. The number (frequently over ten) and the staining intensity of isoesterase bands varies depending on plant species, tissue, and developmental stage (Buschbeck & Zelmer, 1979; Jaaska, 1975).

Glutamic oxaloacetic transaminase- Glutamic oxaloacetic transaminase (GOT) performs a significant role in transamination reactions leading to the elimination of nitrogen from amino acids and the formation of Keto acid for the Kreb's cycle and gluconeogenesis. Although ,the preferred name for this enzyme is aspartate aminotransferase (AAT) and the enzyme is occasionally listed as transaminase (Macdonald and Brewbaker , 1972), it is most frequently referred to as GOT.

All GOT isozymes studied in maize are dimers and are encoded by three loci, GOT1, GOT2, and GOT3 (Scandalios *et al.* 1975; Stuber & Goodman, 1979). Scandalios *et al.* (1975) have reported that products of GOT1 are expressed in the glyoxysomes and products of GOT3 are expressed in mitochondria. They also reported that products of GOT2 are expressed in the soluble fraction.

Three major independent AAT isozymes could be distinguished in seedling zymograms of rye species (Jaaska, 1975, 1981) and triticales (Jaaska & Jaaska, 1976; Tang & Hart, 1975). The same three AAT systems have also been described in wheat and goatgrass (*Acgilops* L.) species (Hart, 1975; Jaaska & Jaaska, 1976). Jaaska (1976, 1981) have designated AAT-A, AAT-B and AAT-C in the decreasing order of their electrophoresis mobility.

Peroxidase- The role of peroxidase has been suggested that it may inhibit growth by mediating the oxidation of indole acetic acid (IAA). Also, many of the peroxidase are associated with cell wall fractions and may be involved in lignification (Mc Cune, 1961; Brewbaker & Hasegawa, 1975). These comprises a large group of

hemo-protein oxidoreductases catalyzing the electron transfer from an oxidizable substrate (phenols, aromatic amines etc.) to hydrogen peroxide. Peroxidases in plants are often encoded by many different loci and show evidence of post-translational modification. Moreover, changes in the number and staining intensity of isoperoxidase bands are often unstable over seedling development and depending on the experimental condition. Five to seven major anodal isoperoxidase were reported by Jaaska (1972, 1975) on polyacrylamide gel. Thirteen major peroxidase isozyme bands (three cathodal and ten anodal) have been identified in maize when subjected to electrophoresis on acrylamide gel.

Superoxide dismutase (SOD) – This is an enzyme catalyzing dismutase of free superoxide radical (O_2^-), generated in a number of oxidative process, in to molecular oxygen and hydrogen peroxide. In the tissue of rye seedling and of related Triticeae grasses, three SOD heterozymes of different molecular structures, biochemical properties and intracellular localization have been identified (Jaaska & Jaaska, 1982). Baum & Scandalios (1979, 1981, 1982) have reported that in maize soperoxide dismutase is compartmentalized in the chloroplasts, mitochondria and cytosol, and have established that genetic control of three of the four sets of SOD isozymes resides in the nucleus. They have named the sets of isozymes SOD 1, SOD 2, SOD 3 and SOD 4 in order of their electrophoretic migrations.

2.10. PROTEIN PAGE ANALYSIS

Electrophoresis is widely used to separate and characterize proteins by applying electric current. Electrophoretic procedures are rapid and relatively sensitive requiring only micro-weights of proteins. Electrophoresis in the polyacrylamide gel is more convenient than in any other medium such as paper and starch gel. Electrophoresis of proteins is carried out in buffer gels containing non denaturing polyacrylamide gels. Separation in buffer gels relies on both the charge and size of the protein where as it depends only upon the size in the SDS gels. Analysis and comparison of proteins in a large number of samples is easily made on polyacrylamide gel slabs.

The seed protein profile has been found to be a characteristic feature of species in several groups of plants (Johnson & Hall, 1965; Johnson & Thein, 1970; Mc.Dainel, 1970). This character sometimes is so sensitive that it can differentiate between types which are morphologically indistinguishable, but genetically divergent from one another (Johnson *et al.*, 1967). Seed protein electrophoresis is increasingly being utilized as an additional approach for species identification and as a useful tool for tracing back the evolution of various group of plants (Ladizinsky & Hymowitz, 1979). These features of the seed protein profile make it a suitable tool in evolutionary studies either for conformation of already established concepts or to resolve controversial problems. Fox *et al.* (1964) found in comparison of a few leguminous species that the protein band pattern of several leguminous species within a genus resembled one another more closely than did those belonging to different genera.

MATERIALS & METHODS

3. MATERIAL AND METHODS

The present study involved accessions of 26 species of *Trifolium*. The details of procurement of material, methodology for morphological data recording, cytological investigation, biochemical analysis, crossability studies and analysis of data is given here under:

3.1 Procurement of germplasm

In an ongoing *Trifolium* improvement programme at IGFRI, Jhansi, accessions of different *Trifolium* species were procured through various sources after carefully searching the available literature and database of various research agencies. Exotic species were procured through NBPGR, India after the necessary official formalities including quarantine check and accessioning. Indigenous *Trifolium* sp. were also procured from different research Institution/ Universities of India including IGFRI, Jhansi. Besides these, many advanced breeding lines and natural as well as induced mutants / variants have been developed in *Trifolium* improvement project of IGFRI.

The material for present study has been taken from *Trifolium* improvement programme. Exotic as well as indigenous accessions of different species, natural and induced mutants, morphological variants, different ecotypes, advanced breeding lines and released varieties of *T. alexandrinum* were selected for this study. The details of various accessions of *Trifolium* used in the present study are given in Table 3.1 and 3.2.

3.2 Raising of material

The healthy seeds of different accessions of *T. alexandrinum* and other *Trifolium* species (exotic as well as indigenous) were sown in nursery and Central Research farm of IGFRI, Jhansi in the second week of October for two consecutive years in 1998 and 1999. Sowing was done in 3m rows at 50 cm distance. Plant to plant distance was maintained at 15 cm. For prostrate species like *T. subterraneum* and *T. repens* row to row distance was maintained at 100 cm and plant to plant distance at 50 cm. Irrigation and fertilizers were applied as per recommended agronomic practices. The first cut of *T. alexandrinum* was taken at 45 days after

Table 3.1. List of different accessions of *Trifolium* species

S. N.	Species	Accession number
1	<i>T. apertum</i>	EC 401712
2	<i>T. resupinatum</i>	SH 98-36, SH 98-72, SH 98-73, SH 98-86, SH 98-15, JHS-3, SH-99-29, SH-99-65, SH-99-23, SH-99-33, SH-99-32, SH-99-25, SH-99-26.
3	<i>T. glomeratum</i>	EC 401700, EC 402170, EC 425033
4	<i>T. subterraneum</i>	EC 401718, EC 401717, EC 402167, IG 96-112, IG 96-112
5	<i>T. vesiculosum</i>	EC 401716, EC 402168
6	<i>T. hybridum</i>	EC 401702, EC 401701, EC 425029, EC 425030, EC 425032,
7	<i>T. repens</i>	EC 401708, EC 400985, EC 400986, EC 400987, EC 400984, EC 401705, EC 401704, EC 401706, EC 401707
8	<i>T. pratense</i>	EC 401721, EC 401720, EC 401719, EC 400735, EC 400982, PRC-3, EC 400980, EC 400979, EC 402168
9	<i>T. incarnatum</i>	EC 402164, IG 96-111
10	<i>T. hirtum</i>	EC 402153, EC 425039, EC 425037, EC 425038, EC 425045
11	<i>T. diffusum</i>	EC 402163
12	<i>T. spumosum</i>	EC 402160
13	<i>T. lappaceum</i>	EC 402165
14	<i>T. argutum</i>	EC 402154
15	<i>T. arvense</i>	EC 402156
16	<i>T. campestre</i>	EC 402155, EC 425028, EC 425026, EC 425027
17	<i>T. constantinopolitanum</i>	EC 401713
18	<i>T. cherleri</i>	EC 401703
19	<i>T. nigrescens</i>	EC 425049, EC 425047, EC 425048
20	<i>T. echinatum</i>	EC 401714, EC 425076, EC 425075, EC 425077, EC 425078
21	<i>T. medium</i>	EC 425045
22	<i>T. alpestre</i>	EC 425043, EC 425042
23	<i>T. tembense</i>	EC 425064, EC 425065, EC 425066, EC 402169
24	<i>T. purpureum</i>	EC 425069, EC 425070
25	<i>T. angustifolium</i>	EC 425062, EC 425061
26	<i>T. retusum</i>	EC 402150

Table 3.2. List of different lines of *T. alexandrinum* used in study

S.N.	Genotype	S.N.	Genotype
1	EC 329299	36	Raj 7/53-54 -2
2	IL 40010	37	Raj 7/13-14- O
3	EC 400733	38	JHTB 5-90-I
4	EC 401710	39	IL 40010-Mes
5	EC 401709	40	JHB 94 -18/11
6	Wardan	41	JHB 91 P-20
7	EC 402161	42	JHB 34/22
8	EC 400977	43	Raj - Bundi - O
9	EC 400976	44	JHB -P- 23/35
10	EC 401711	45	JHTB -1-90-A1
11	JHB 94 P-22	46	JHB 94 -31
12	JHB 94 -R-16	47	JHB 94 -25
13	JHB 94 -R-35	48	IL 40014
14	JHB 94 -R-13	49	IL 40013
15	JHB 94 -R-25	50	JHB 94-P-60
16	JHB 94 P/T -34	51	BL 144
17	EC 318951	52	JHB94-56
18	JHB 57P3	53	BL 142
19	JHB P17-1	54	Raj 7/13-25
20	Raj 7/13-14	55	HFB 155
21	JHB 15-27	56	BL 131
22	JHB6/54 p/t	57	JHB 36/5-54
23	JB 92-1	58	JHB CT2 6/35
24	BL 122	59	JHB 6/54
25	JHB 146	60	JHB 16/2
26	Raj 7/49-50	61	HFB 155
27	JHTB 9-90 N1	62	Wardan S-1
28	JHB 5-13/12	63	Wardan S-2
29	IL 4009	64	Wardan S-3
30	JHTB 5-90-2	65	Wardan S-4
31	JHTB 3-90-H	66	JHB 99-32-1
32	JHTB 13-90-B	67	JHB 99-25
33	Raj 7/53-54	68	JHB 99-32-2
34	JHTB-1-90-P3	69	JHB 99-32-3
35	Raj 7/53-54- O		

sowing and subsequent cuttings were taken at 30 days interval. Cuttings of other *Trifolium* species were taken as per their growth in local conditions which varied from species to species.

3.3 Morphological observations

Morphological observations were recorded for following characters at 50% flowering stage. The morphological observation were grouped in qualitative and quantitative traits:

3.3.1. Qualitative Traits

Habit : Habit of the plant was recorded as erect, semi erect and prostrate.

Hairiness : The hairiness of stem, stipule, petiole and leaflet was recorded as dense hairy (DH), medium hairy (MH), non-hairy (NH) or glabrous.

Colour : Colour of petiole, stem, stipule and leaf were recorded as light green, dark green, violet green, reddish green etc.

Leaflet shape : Leaflet shape was recorded according to standard morphological nomenclature such as round, elliptical, ovate/obovate, oblong etc.

Leaf margin : Leaf margin was recorded as entire / serrate/ dentate etc.

Flower characters : Observations on date of initiation of flowering, flower colour, date of 50% flowering, mature inflorescence length and mature inflorescence width were noted.

3.3.2. Quantitative traits

Data on certain quantitative traits such as plant height, leaf length and breadth, stipule length etc. was recorded at 50% flowering stage. For quantitative traits data was recorded from three plants in each line. Range and mean of different observations were calculated. The characters included were as under:

Plant height/ rosette diameter : The plant height was measured in erect species from the base to the top of the plant in centimeter (cm). Rosette diameter in prostrate species was measured in three directions and mean of it was noted in centimeter (cm).

Branching : Total number of branches per plant were recorded.

Number of leaves: Total number of leaves per plant were recorded.

Leaflet length : Leaflet length of middle leaflet of third leaf from the top was

measured in centimeter (cm).

Leaflet breadth : Leaflet breadth of middle leaflet of third leaf from the top was measured in centimeter (cm).

Stipule length : The length of stipule was measured separately of fused and free portion in centimeter (cm).

Petiole length : The petiole length was measured from the axil to the leaf base in centimeter.

3.4. Cytological studies

3.4.1. Meiotic studies

Meiotic studies were carried out in pollen mother cells (PMCs) from young flower buds. The most suitable time for collection of the flower buds was found to be between 8 AM to 9 AM. The flower buds were fixed in Glacial acetic acid : Ethanol (1:3) for 24 hours.

The anthers were taken out from the fixed flower buds and smeared in a drop of 2% aceto-carmin solution on a clean glass slide. All the stages of meiosis right from prophase meiosis I to the pollen formation were observed under Nikon Labophot 2 light microscope. The cytological analysis were made from temporary slide and suitable cells were photographed on NOVÅ Black and White (125 ASA) film with Nikon FX-35 Dx microphotographic camera.

3.4.2. Pollen stainability

Pollen grains from dehiscent anthers were collected on slides and stained in a solution of 2% acetocarmine : glycerol (1:1) for one hour. Pollen grains which were plump, round and deeply stained were scored as viable, while shriveled and non-stained ones were recorded as nonviable. Each slide was prepared using anthers from three flowers of one inflorescence and approximately 500 pollen grains were scored per slide. Three slides were prepared for each plant.

3.5. Isozyme studies

T. alexandrinum and other *Trifolium* species were compared for the five enzymes- Peroxidase (Per, E.C.1.11.1.7), Esterase(Est, E.C.3.1.1.2), Superoxide dismutase (SOD, E.C.1.15.1.1), Acid phosphatase (ACP, E.C.3.1.3.2) and Glutamate oxalo acetate transaminase (GOT or AAT, E.C.2.6.1.1).

Horizontal starch gel electrophoresis technique of Smithies (1955) with discontinuous buffer system of Poulik (1957) was used for studying the enzyme systems, because it has a better resolving power than other techniques (Brewer & Singh, 1972) which results from the gradation of molecular sieving effect of starch gel matrix besides electrophoretic separation.

3.5.1. Preparation of enzyme extract

The crude extract of young, green and healthy leaves was prepared by homogenising 1g of sample with 0.3 ml of chilled tris buffer (pH 8.65), using pre-chilled pestle and mortar. Care was taken to collect leaf samples at same stage of growth from different accessions. The crude extract was filtered through muslin cloth and the filtrate was stored in the deep fridge (-20° C) in different vials and thawed just before use.

3.5.2. Preparation of the gel

Out of many concentrations of starch tried, 14% starch was found suitable for adequate polymerization and the gel prepared was translucent. 22.4 g of hydrolysed potato starch was taken in a conical flask and 160 ml gel buffer was added to it. The content was heated and was vigorously shaken while heating, till it became less viscous and translucent. The cooked starch was immediately poured on to a glass plate (12 cm x 12 cm) having 4 tiers of glass strips pasted on it. The tray was then covered with a glass plate in such a way that air bubbles were prevented from being trapped. The gel plate was kept to set at room temperature for 5 to 6 hrs.

3.5.3. Application of samples

The cover from gel plate was removed with the help of a blade. Slots were made in the middle of the gel for peroxidase and proximally for the other 4 enzymes with the help of the stick cutter. The samples to be analyzed were soaked onto filter paper wicks (Whatman no. 3) of size 5 mm x 8 mm and inserted into the slots.

3.5.4. Electrophoresis

The gel plate was then placed in 'Genei' horizontal migration chamber. Bridge buffer was poured into the buffer chambers so that electrodes were

completely dipped. The plate was connected with the buffer chamber with the help of filter papers (Whatman no.1), which was earlier saturated with buffer.

Electrophoresis was carried out at constant current of 24 mA for first 30 minutes followed with 34 mA till the front reached the other end. During the process, the migration chamber was placed in a refrigerator to avoid overheating of the gel. The gel plate was taken out and the glass strips were removed from the sides of the glass plate. Each gel was sliced thrice, horizontally, with the help of a copper wire, to obtain four slices of gel. The first slice was rejected and lower ones were used for staining.

3.5.5. Staining of the gel

The gel was stained for peroxidase following the method given by Veech (1969) and for esterase, GOT, SOD and ACP following Wendel and Weeden (1989).

Peroxidase : 100 mg of benzidine was dissolved by heating in 100 ml of 0.2 M acetate buffer (pH 5.6). In 100 ml of benzidine solution, 2 ml of 3% hydrogen peroxide was added at the time of incubation of the gel. After 10 minutes of incubation, blue bands appeared which turned brown later. The sites of peroxidase isozymes were stained by the oxygen released in the reaction which oxidises benzidine, a colourless compound to a coloured one.

Esterase : The gel was incubated in 100 ml of 0.1 M phosphate buffer (pH 6.5) containing 32.5 mg of 1-naphthyl acetate in 1 ml acetone and 50 mg of fast blue RR salt at room temperature for 60 minutes. The site of esterase enzyme activity appeared as reddish brown to blackish bands on the gel.

Acid phosphatase : The gel was incubated in 100 ml of acetate buffer (pH 5.6) containing 100 mg of Na-1-naphthyl phosphate, 100 mg $MgCl_2$ and 100 mg of fast blue RR salt at 37°C in the dark until desired staining intensity of bands has occurred.

Aspartate Amino Transferase (AAT or GOT): The gel was stained in 100 ml of AAT substrate (for composition see section preparation of reagents and buffers

below) solution containing fast blue BB salt (100 mg). The gels were incubated in dark at room temperature until blue bands appear.

Superoxide Dismutase: The gel was stained in 100 ml of Tris-HCl buffer (pH-8.65), in which Riboflavin (4 mg) EDTA (2mg) and NBT (20 mg) were added. The gel was incubated in dark for 30 minutes and then exposed to intense light for 1.5 hrs until bands appear.

3.5.6. Preparation of Zymograms

The bands for different isozymes were drawn on graph sheet at 1:1 ratio. The point of origin and front was also marked in order to calculate relative mobility of the bands. Selected gel plates were also photographed.

3.5.7. Nomenclature of bands

The bands were scored from the place of origin where samples were loaded to the end of front movement. The slowest band was considered as Band 1 and subsequently bands were numbered based on the pooled zymogram for the genus. In case of Peroxidase bands were named separately as cathodal and anodal bands and in both cases the movement of bands was considered from the place of origin where the samples were loaded.

3.5.8. Preparation of reagents and buffers:

Gel buffer (pH - 8.65) - Tris citrate buffer: 9.206 g Tris and 1.051 gm Citric acid were dissolved in distilled water and final volume was made up to 1 litre.

Bridge buffer (pH- 8.65) Sodium borate buffer: 18.550 g of boric acid and 4.0 g of NaOH dissolved in distilled water and final volume was made up to 1 litre.

Acetate buffer (pH 5.6): 27.216 mg sodium acetate trihydrate and 2.6 ml of acetic acid dissolved and volume made up to 1000 ml of distilled water.

Phosphate buffer (pH - 6.5):

(a) Mono basic - 31.2 g of sodium dihydrogen orthophosphate dissolved in 1000 ml of distilled water.

(b) Dibasic- 28.4 g of disodium hydrogen orthophosphate dissolved in 1000 ml of distilled water.

(c) For Esterase staining 68.5 ml of monobasic phosphate buffer, 31.5 ml of dibasic phosphate buffer and 100 ml distilled water was mixed to make Phosphate buffer (pH 6.5).

AAT substrate solution (pH- 7.4): The following quantities of chemicals were dissolved in H₂O and volume was made up to 100 ml.

α - ketoglutaric acid (37 mg), L- Aspartic acid (134 mg), PVP-40 - (500 mg), EDTA, Na₂ salt (50 mg), Sodium phosphate dibasic (1.45 g)

Details of important chemicals used in the study

Chemical	Make	Catalogue number	Molecular weight
1- Naphthyl acetate	Loba Chemie	4785	186
Fast Blue RR	BDH	28377	
NBT	SRL	144928	817.65
Riboflavin	HiMedia	RM 181	376.37
EDTA	Hi media	RM 678	292.25
α ketoglutaric acid	Hi media,	RM-245	146.1
L Aspartic acid	Hi media	RM-083	33.10
PVP-40	SRL	164798	40,000
EDTA, Na ₂ salt	Qualigens	18454101	372.24
Sodium phosphate dibasic	Loba Chemie	5972	141.96
Fast blue BB	Hi media	RM-814	RM-814
Sodium 1- Naphthyl phosphate	Loba Chemie	5945	264.15
Tris	SRL	2044122	121.14
N-N-N-N- Tetramethyl ethylendiamine (TEMED)	SISCO	202788	116.21
Acrylamide	SISCO	014022	71.08
N-N Methylene Bis acrylamide	SISCO	134985	154.17
Ammonium Per Sulfate	SISCO	0148134	228.20

3.6 Protein analysis

3.6.1. Polyacrylamide gel electrophoresis (PAGE) analysis for protein

PAGE analysis was done using Polyacrylamide vertical gel electrophoresis system.

3.6.1.1. Preparation of stock solution and buffers

A: Acrylamide stock solution (Separating gel): Acrylamide stock solution for separating gel was prepared by dissolving 29.2 g Acrylamide and 0.8 g of Bis-acrylamide in double distilled deionized water and final volume was made up to 100 ml. Solution was stored at 4°C in amber coloured bottle.

B: Tris HCl buffer for separating (resolving gel) (pH 8.9): 18.15 g of Tris was dissolved in 60 ml of water and pH was adjusted to 8.9 by adding drops of 1N HCl and final volume was made up to 100 ml using deionized double distilled water.

C: Tris HCl buffer for stacking gel) (pH 6.7): 6.1 g of Tris was dissolved in 60 ml water and pH was adjusted to 6.7 by adding drops of 1N HCl and final volume was made up to 100ml.

D: Ammonium per sulphate solution (APS): 0.15 g Ammonium per sulphate was dissolved in 100 ml distilled water. This solution was prepared fresh each time.

E: Riboflavin solution: 0.4 mg Riboflavin was dissolved in 10 ml distilled water and was filtered before use.

F: Running gel electrode buffer (pH 8.3): Electrode buffer (pH 8.3) was prepared by dissolving 0.6 g Tris and 2.8 g of Glycine in 1 litre distilled water.

G : Tracking dye : Tracking dye was prepared by mixing 0.25 % Bromophenol blue with 40% Sucrose solution.

3.6.1.2. Preparation of resolving gel

40 ml 12% resolving gel was prepared by adding Acrylamide (30%, 16 ml), Tris HCl (10 ml), H₂O (14 ml), TEMED (20 μ l) and Ammonium per Sulfate (10%, 200 μ l). The gel solution was immediately poured in vertical gel casting unit and left for one hour for setting in undisturbed condition. At the top of resolving gel 5 ml of water was poured so that the gel does not get dried.

3.6.1.3. Preparation of stacking gel

10 ml of 4% stacking gel was prepared by adding Acrylamide (30%, 1.3 ml), Tris HCl (2.5 ml), H₂O (6.2 ml), TEMED (10 μ l) and APS (10%, 50 μ l). This gel solution was poured over the resolving gel after removing the top level of water. Pouring of staking gel was immediately followed with placing of comb. This gel was left overnight. 50 micro litre of each sample was mixed with 4 micro liter of tracking dye and was loaded in different wells after removing the comb.

3.6.1.4. Electrophoresis

The gel plate thus prepared was placed in 'Genei' vertical migration chamber. Running gel electrode buffer was poured into the migration chambers so that electrodes were completely dipped. The plate was connected with the buffer chamber.

A constant current of 20 mA was given till the tracking dye crossed the stacking gel portion. After that current was increased to 40 mA till the tracking dye reached the bottom of the gel.

3.6.1.5. Staining

After running the gel, it was fixed in 3.5 % Perchloric acid prepared by dissolving 5ml of Perchloric acid (70%) in 95 ml distilled water for overnight. Following day the fixative was removed and stained in staining solution prepared by dissolving 40 mg of comossie brilliant blue G-250 in 100ml 3.5 % Perchloric acid for 3 to 4 hours till desired intensity of bands observed.

3.6.1.6. Preparation of zymogram

The different protein bands were drawn on graph sheet at 1:1 ratio. The point of origin was marked in order to see the relative mobility of the bands. Selected gel plates were also photographed.

3.6.1.7. Scoring and nomenclature of bands

The bands were scored from starting point *i.e.* the well where the samples were loaded. The slowest band was treated as first band and the bands were numbered on the basis of pooled zymogram prepared for the genus.

3.6.2. Crude protein estimation

Ten lines of berseem genotypes were selected for quantitative protein content. The selected lines were harvested on 25th day of regeneration after first cut. Representative samples of whole plant (leaf and stem) were collected and sun dried.

After grinding, the samples were analysed in duplicate for crude protein following recommended method (AOAC, 1980).

3.7. Analysis of data

3.7.1. Cluster analysis based on morphological characters

Numerical data recorded for various morphological traits were analyzed using non-hierarchical Euclidian cluster analysis for grouping of genotypes (Spark, 1973). All computation were done using the computer software SPAR 1 Release 1.1 (IASRI, New Delhi). The programme was used to generate principal components scores data. The replicated data were averaged over replicates and then cluster analysis was performed.

3.7.2. Similarity matrix analysis and cluster based on isozyme bands

A binary data matrix reflecting the presence or absence of specific isozyme band was generated for all the accessions of *Trifolium* species. Only unambiguously scored bands were used in the matrix. Binary data matrix using isozyme banding pattern data has also been followed by other workers (Pisupati, 1999).

The genetic similarities (GS) between line i and j were estimated using the formula of Dice (1945) $G_{sij} = 2N_{ij} / (N_i + N_j)$,

Where, N_{ij} is the number of common bands between i and j and N_i is the number of bands in i and N_j is the number of band in j respectively.

A dendrogram was generated using the unweighted-pair-group method average (UPGMA) clustering procedure. All computations were done using the computer software NTSYS-PC version 1.60.

3.7.3. Estimate of variability for isozyme banding pattern

The number of observed strains differed from species to species. Hence, estimate of variability for zymogram pattern was calculated as per method given by Shahi *et al.* (1969). If a species has N zymograms distributed among its strains with equal frequency and n strains are randomly chosen, the number of zymograms to be

found among them will be $N\{1 - (1 - 1/N)^n\}$ and its standard deviation will be $\sqrt{n N [1/N(1-1/N)]}$. On this expectation an estimate of variability given by the standardized value of the expected number of zymograms was computed for each species.

3.8. Interspecific hybridization

3.8.1. Pollination

Interspecific crosses were attempted using emasculation followed by pollination method. Suitable flower buds were emasculated prior to their opening (prior to anthers dehiscence). Anthers were removed with the help of a forceps and needle by opening their keels from one end in the morning (between 7am to 9 am). The pollen grains taken from freshly opened flowers of desired male parent were applied gently on the stigma of emasculated flower with the help of needle. After pollination the flowers were covered with butter paper bags to check further pollination. Pollinated flower were properly labeled. Regular monitoring of pollinated flowers for various fertilization indicators such as withering of floral parts, development of ovules *etc.* was done.

Some flowers were left as such in order to see the seed set under natural condition whereas from some of the pollinated flowers embryos were excised 5-6 days after pollination and transferred on suitable culture media in artificial condition.

3.8.2. Embryo excision

Suitable time for embryo excision was determined. Flowers were taken to laboratory after 1 to 10 days after pollination (DAP) regularly and examined under stereoscopic microscope. It was found that embryo could be differentiated from the maternal tissues only after 5-6 DAP in different cases. After 8 DAP, the embryo development stops and embryo aborts if left in field condition. On this basis, the ideal time for embryo excision was found to be 5-6 DAP in all the cross combinations involving *T. alexandrinum* as the female parent.

3.8.3. Media preparation and embryo culture

MS (Murashige and Skoog, 1962) and L2 basal (Phillips and Collins, 1984) inorganic media with varying levels of hormonal concentrations were used with minor modifications for standardizing the embryo culture protocol.

MS Basal and L2 Basal media was prepared by mixing various inorganic and organic salts in double distilled water (as given in table 3.3). Different combinations of growth hormones were added at mentioned dosages before the addition of agar. pH of the media was adjusted to 5.8 with 1N NaOH. After the addition of agar the medium was poured into test tubes and plugged with non-absorbent cotton wrapped in muslin cloth. The medium was autoclaved for 25 minutes at 15 psi pressure. Tubes containing autoclaved medium were left for overnight for solidification at room temperature. After the solidification the medium was used for ovule/embryo culture.

3.8.4. Ovule/embryo culture

Pollinated flowers were brought to Laboratory 5-6 days after pollination. The ovules were excised, surface sterilized under aseptic conditions and put in the culture media. The cultures were maintained under standard conditions ($25 \pm 2^{\circ}$ C) under dark condition till embryo germination. After embryo germination the plants were provided with 16 /8 hours of photoperiod.

Table 3.3. Composition of L2 and MS basal media and different embryo culture media.

S N	Components	L2 basal	MS basal
1	KNO ₃	20.8 mM	18.8 mM
2	NH ₄ NO ₃	12.5 mM	20.6 mM
3	KH ₂ PO ₄	2.34 mM	1.25 mM
4	MgSO ₄ . 7H ₂ O	1.8 mM	1.5 mM
5	CaCl ₂ . 2H ₂ O	4.1 mM	3.0 mM
6	NaH ₂ PO ₄	0.6 mM	-
7	FeSO ₄ .EDTA. 7 H ₂ O	90 µM	100 µM
8	Na ₂ EDTA. 2H ₂ O	-	100 µM
9	MnSO ₄ . 4 H ₂ O	90 µM	100.0 µM
10	H ₃ BO ₃	82 µM	100.0 µM
11	ZnSO ₄ . 7H ₂ O	18 µM	30.0 µM
12	KI	6 µM	5.0 µM
13	Na ₂ MoO ₄ . 2H ₂ O	1.7 µM	1.03 µM
14	CoCl ₂ . 6H ₂ O	0.42 µM	0.105 µM
15	CuSO ₄ . 5 H ₂ O	0.4 µM	0.1 µM
16	Myo-inositol	1.4 µM	100 mg/L
17	Thiamine HCl	6 µM	0.1 mg/L
18	Pyridoxine HCl	2.4 µM	0.5 mg/L
19	Nicotinic acid	-	0.5 mg/L
20	Sucrose	73 mM	87.6 mM
21	Agar	0.8 %	1.0 %
22	pH	5.8	5.8

Composition of different embryo culture media

EC1= L2 basal + 0.001mg/l 2,4 D + 3.225 mg/l adenine + 2.5% sucrose

EC2= L2 basal + 0.006mg/l NAA + 2.0 mg/l adenine + 12.5% sucrose

EC3= MS basal + 0.5 mg/l Kinetin + 3.0% sucrose

EC4= L2 basal + 32.442 mg/ L adenine + 0.0465 mg/L NAA + 12.5% sucrose

EC5= MS basal + 0.5mg/l Kinetin + 12.5% sucrose

RESULTS

4. RESULTS

The work was conducted under following heads:

- 4.1 Morphological studies
- 4.2 Cytological studies
- 4.3 Isozyme studies
- 4.4 Qualitative and quantitative protein studies
- 4.5 Interspecific compatibility

4.1 Morphological studies

4.1.1 Intra species variation

Data on various morphological traits were recorded in 25 *Trifolium* species. The data are presented in Tables 4.1 to 4.5 and details of observations on each species including intra species variation is described below:

T. hirtum:

The plants were annual and prostrate/rosette in habit, attaining a diameter ranging from 15.3 cm to 17.7 cm. Profuse primary and secondary branching along with large number of leaves gave a compact rosette appearance. The average number of primary branches was 14.2 per plant, while number of leaves per plant varied from 95 to 183 with an average of 130. Stem was scanty hairy. Petiolate leaves were alternately attached to stem. Petioles were with medium to dense pubescence and reddish green in colour. Leaflets were obovate / elliptical in shape and green to dark green in colour, leaf margin was entire in two accessions and serrate in EC 425039. Leaflet length and breadth ranged from 1.1 to 1.3 cm and 1.0 cm to 1.1 cm respectively. Stipules were narrow and ovate in shape whose fused portion was 0.8 cm long whereas free portion was 0.57 cm long. In Jhansi condition the flowering initiation was noted in 4th week of March in EC 402153 and EC 425039 while in EC 425037 it was observed in the 3rd week of April.

T. diffusum:

The plants were annual and prostrate in nature. The plants were spreading rosette, with diameter ranging from 13.8 cm to 19.7 cm. The average number of primary branches were 7.3 per plant and the number of leaves per plant ranged from

Table 4.1: Morphological characters of accessions of different <i>Trifolium</i> species								
Species	Accn. No.	ch1	ch2	ch3	ch4	ch5	ch6	ch7
<i>T. hirtum</i>	EC 402153	P	RG	MD	OBO	Et	G	GL
<i>T. hirtum</i>	EC 425039	P	RG	HD	OBO	Sr	D G	HD
<i>T. hirtum</i>	EC 425037	P	RG	HD	ELL	Et	D G	HD
<i>T. diffusum</i>	EC 402163	P	G	LD	OBO	Et	G	MD
<i>T. spumosum</i>	EC 402160	P	G	GL	RHO	Sr	G	GL
<i>T. lappaceum</i>	EC 402165	P	G	GL	OBO	Et	D G	MD
<i>T. argutum</i>	EC 402154	P	RG	GL	OBO	Sr	G	GL
<i>T. arvense</i>	EC 402156	SE	RG	GL	OBO	Sr	G	GL
<i>T. compestre</i>	EC 402155	P	VG	GL	OBO	Et	G	GL
<i>T. compestre</i>	EC 425028	P	G	MD	ELL	Sr	LG	GL
<i>T. compestre</i>	EC 425026	SE	RG	LD	OBO	Sr	G	GL
<i>T. compestre</i>	EC 425027	P	VG	LD	ELL	Et	G	GL
<i>T. apertum</i>	EC 401712	P	VG	MD	OBO	Et	D G	GL
<i>T. constantinopolitanu</i>	EC 401713	P	RG	LD	ELL	Et	G	LD
<i>T. repens</i>	EC 401708	P	VG	GL	RHO	Sr	G	GL
<i>T. repens</i>	EC 400985	P	VG	GL	RHO	Sr	D G	GL
<i>T. repens</i>	EC 400986	P	RG	GL	RHO	Et	D G	GL
<i>T. alexandrinum</i>	EC 401711	E	VG	MD	ELL	Et	G	MD
<i>T. alexandrinum</i>	WARDAN	E	G	LD	ELL	Et	G	MD
<i>T. alexandrinum</i>	EC 400976	E	VG	LD	ELL	Et	G	MD
<i>T. alexandrinum</i>	EC 400977	E	G	MD	ELL	Et	G	GL
<i>T. alexandrinum</i>	EC 402161	E	VG	MD	ELL	Et	G	MD
<i>T. alexandrinum</i>	JHB146	E	G	LD	ELL	Et	D G	LD
<i>T. alexandrinum</i>	EC 401709	E	VG	LD	ELL	Et	D G	MD
<i>T. alexandrinum</i>	EC 401710	E	G	MD	ELL	Et	D G	MD
<i>T. alexandrinum</i>	EC 400733	E	G	LD	ELL	Et	D G	LD
<i>T. pratense</i>	EC 401721	E	RG	LD	RHO	Et	D G	GL
<i>T. pratense</i>	EC 401720	E	RG	GL	OBO	Et	D G	GL
<i>T. pratense</i>	EC 401719	E	VG	GL	OBO	Et	D G	LD
<i>T. pratense</i>	EC 400735	E	RG	HD	OBO	Et	D G	HD
<i>T. pratense</i>	EC 400982	E	RG	HD	OBO	Et	D G	MD
<i>T. pratense</i>	PRC_3	E	RG	HD	OBO	Et	G	MD
<i>T. pratense</i>	EC 400980	E	RG	HD	ROU	Et	G	HD
<i>T. pratense</i>	EC 400979	E	RG	HD	OBO	Et	G	MD
<i>T. pratense</i>	EC 402168	E	RG	HD	OBO	Et	G	GL
<i>T. hybridum</i>	EC 401702	SE	RG	GL	ROU	Sr	G	GL
<i>T. hybridum</i>	EC 401701	SE	RG	GL	ROU	Sr	G	GL
<i>T. hybridum</i>	EC 425029	SE	VG	GL	OBO	Sr	G	GL
<i>T. hybridum</i>	EC 425030	SE	VG	GL	OBO	Sr	D G	GL
<i>T. hybridum</i>	EC 425032	E	RG	GL	ELL	Sr	D G	GL

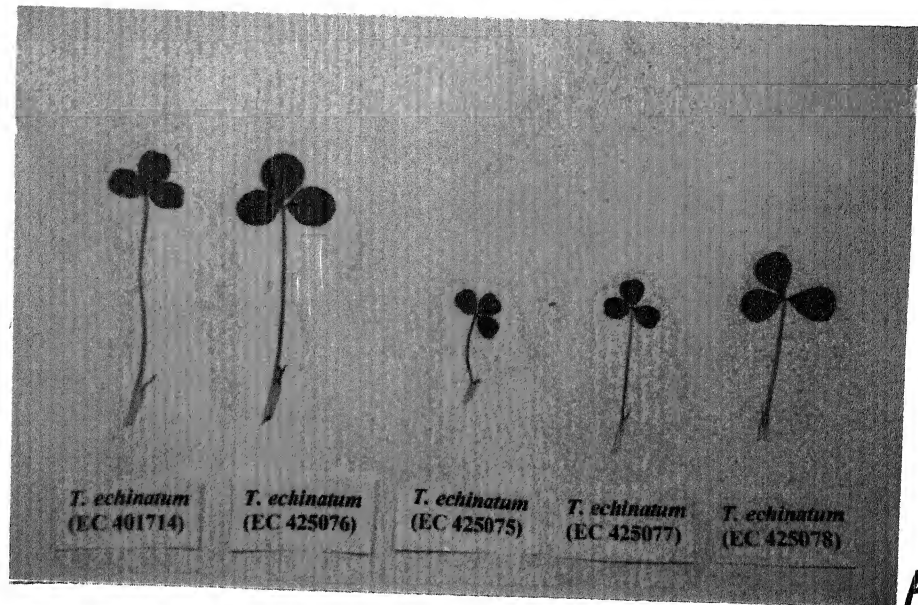
Table4.1 (Contd.): Morphological characters of accessions of different <i>Trifolium</i> species								
Species	Accn. No.	ch1	ch2	ch3	ch4	ch5	ch6	ch7
<i>T. subterraneum</i>	EC 401718	P	RG	MD	OVA	Et	G	LD
<i>T. subterraneum</i>	EC 401717	P	RG	HD	OVA	Et	G	HD
<i>T. subterraneum</i>	EC 402167	P	RG	LD	OVA	Et	G	LD
<i>T. subterraneum</i>	c.v. Bachus Mar	P	RG	MD	OVA	Et	G	MD
<i>T. subterraneum</i>	c.v. Claire	P	RG	MD	ELL	Et	G	GL
<i>T. glomeratum</i>	EC 401700	P	RG	GL	OBO	Sr	LG	GL
<i>T. glomeratum</i>	EC 402170	SE	G	GL	OBO	Sr	LG	GL
<i>T. glomeratum</i>	EC 425033	E	G	GL	OBO	Sr	LG	GL
<i>T. incarnatum</i>	EC 402164	SE	G	MD	OBO	Et	G	HD
<i>T. incarnatum</i>	IG 96-111	SE	G	MD	OBO	Et	G	GL
<i>T. cherleri</i>	EC 401703	P	G	HD	OBO	Sr	D G	HD
<i>T. nigrescens</i>	EC 425049	P	VG	LD	OBO	Et	D G	MD
<i>T. nigrescens</i>	EC 425047	P	G	LD	ROU	Sr	D G	LD
<i>T. nigrescens</i>	EC 425048	P	VG	MD	OBO	Et	G	MD
<i>T. echinatum</i>	EC 401714	P	VG	LD	OBO	Et	G	MD
<i>T. echinatum</i>	EC 425076	P	G	HD	ELL	Et	G	MD
<i>T. echinatum</i>	EC 425075	P	VG	LD	OBO	Et	D G	LD
<i>T. echinatum</i>	EC 425077	P	VG	MD	ELL	Et	G	HD
<i>T. echinatum</i>	EC 425078	E	VG	MD	ELL	Et	D G	HD
<i>T. medium</i>	EC 425045	P	VG	GL	OBO	Et	D G	GL
<i>T. alpestre</i>	EC 425043	P	VG	LD	OBO	Et	D G	LD
<i>T. alpestre</i>	EC 425042	E	VG	MD	OBO	Et	D G	MD
<i>T. tembense</i>	EC 425064	E	RG	GL	ELL	Sr	G	GL
<i>T. tembense</i>	EC 425066	E	RG	GL	ELL	Sr	D G	GL
<i>T. tembense</i>	EC 425065	E	RG	GL	ELL	Sr	D G	GL
<i>T. tembense</i>	EC 402169	E	RG	GL	ELL	Sr	G	GL
<i>T. resupinatum</i>	SH 98-36	P	G	GL	ELL	Sr	G	GL
<i>T. resupinatum</i>	SH 98-72	E	G	GL	ELL	Sr	G	GL
<i>T. resupinatum</i>	SH 98-73	SE	G	GL	OBO	Sr	D G	GL
<i>T. resupinatum</i>	SH 98-86	SE	G	GL	ELL	Sr	D G	GL
<i>T. resupinatum</i>	SH 98-15	P	G	GL	ELL	Sr	D G	GL
<i>T. purpureum</i>	EC 425069	SE	G	HD	ELL	Et	G	HD
<i>T. purpureum</i>	EC 425070	SE	RG	HD	ELL	Et	G	MD
<i>T. angustifolium</i>	EC 425062	SE	G	MD	ELL	Et	G	MD
<i>T. angustifolium</i>	EC 425061	SE	G	MD	ELL	Et	G	GL
Ch1= Habit								
Ch2 = Petiole colour		Ch3= Petiole hairiness						
Ch4 = Leaf shape		Ch5 = Leaf margin		Ch6 = Leaf colour		ch7= leaf hairiness		
P= Prostrate		E= Erect		SE = Semi erect		G= Green		
RG=Reddish Green		VG = Violet Green		LD= Low hair density				
MD = Medium hairy		HD = Dense hairy		Gl+F24= glabrous				
RHO = Rhomboid		OBO = Oblong		ELL = Elleptical		OVA= Ovate		
ROU = Round		Sr = Serrate		Et = Entire				

Table 4.2 : Morphological characters of accessions of different <i>Trifolium</i> species									
Species	Accn. No.	ch1	ch2	ch3	ch4	ch5	ch6	ch7	ch8
<i>T.hirtum</i>	EC402153	11.3	95.0	17.7	6.1	1.3	1.1	1.0	0.5
<i>T.hirtum</i>	EC425039	21.3	183.0	15.9	4.6	1.1	1.0	0.6	0.6
<i>T.hirtum</i>	EC425037	10.0	112.0	15.3	4.4	1.3	1.1	0.9	0.6
<i>T.diffusum</i>	EC402163	7.3	72.0	17.0	6.1	1.7	1.3	1.3	0.6
<i>T.spumosum</i>	EC402160	7.3	59.0	19.8	8.4	1.3	1.3	0.8	0.6
<i>T.lappaceum</i>	EC402165	6.7	35.0	8.1	2.5	0.8	0.7	0.8	0.4
<i>T.argutum</i>	EC402154	6.3	73.3	13.2	5.2	1.1	0.9	0.5	0.5
<i>T.arvense</i>	EC402156	6.0	50.0	20.3	7.7	1.7	1.2	0.8	0.6
<i>T.campestre</i>	EC402155	7.5	59.0	8.8	1.9	0.9	0.8	0.6	0.5
<i>T.campestre</i>	EC425028	18.0	400.0	34.0	1.1	0.8	0.5	0.4	0.2
<i>T.campestre</i>	EC425026	8.3	477.0	39.4	1.1	1.1	0.7	0.4	0.4
<i>T.campestre</i>	EC425027	1.0	17.0	11.0	0.5	1.3	0.7	0.3	0.4
<i>T.apertum</i>	EC401712	11.3	167.0	22.5	7.3	1.6	1.2	1.2	0.8
<i>T.constantinopolitanum</i>	EC401713	13.0	120.0	20.3	7.7	1.2	1.0	0.9	0.5
<i>T. repens</i>	EC401708	7.0	35.0	13.3	5.7	1.3	1.0	0.6	0.5
<i>T.repens</i>	EC400985	5.0	27.0	10.0	4.2	1.0	1.0	1.3	0.7
<i>T.repens</i>	EC400986	5.0	23.0	11.7	4.9	1.3	1.2	1.3	0.7
<i>T.alexandrinum</i>	Wardan	6.7	73.0	43.7	6.7	4.5	1.8	2.1	1.2
<i>T.alexandrinum</i>	EC401711	3.3	30.0	25.3	5.2	3.1	1.4	1.8	0.8
<i>T.alexandrinum</i>	EC400976	3.5	29.0	16.5	5.3	2.4	1.1	1.9	1.0
<i>T.alexandrinum</i>	EC400977	4.0	63.0	5.0	3.8	4.3	1.4	1.7	1.3
<i>T.alexandrinum</i>	EC402161	6.7	76.3	50.7	4.9	4.9	1.7	1.8	1.3
<i>T.alexandrinum</i>	JHB 146	4.0	59.0	33.0	4.2	3.7	1.2	1.8	1.3
<i>T.alexandrinum</i>	EC401709	5.3	66.0	34.7	6.7	3.6	1.7	2.0	1.2
<i>T.alexandrinum</i>	EC401710	6.3	72.0	40.0	6.1	4.1	1.7	2.1	1.3
<i>T.alexandrinum</i>	EC400733	6.5	75.0	32.0	2.4	3.0	1.3	1.1	0.9
<i>T.pratense</i>	EC401721	2.3	11.3	7.4	3.6	1.2	1.1	0.4	0.3
<i>T.pratense</i>	EC401720	3.0	15.0	8.5	3.8	1.3	1.2	0.5	0.3
<i>T.pratense</i>	EC401719	4.0	26.0	17.2	6.7	2.2	1.6	0.9	0.5
<i>T.pratense</i>	EC400735	2.7	12.7	13.0	5.8	1.8	1.6	0.5	0.4
<i>T.pratense</i>	EC400982	4.3	22.3	21.5	7.9	2.7	2.0	1.0	0.5
<i>T.pratense</i>	PRC 3	3.3	14.3	12.6	6.3	1.7	1.5	0.6	0.5
<i>T.pratense</i>	EC400980	4.3	19.3	12.9	6.3	1.7	1.4	0.8	0.6
<i>T.pratense</i>	EC400979	2.7	16.7	11.9	5.4	1.7	1.4	0.5	0.6
<i>T.pratense</i>	EC402168	1.0	8.5	7.0	5.1	1.6	1.4	0.9	0.5
<i>T.hybridum</i>	EC401702	4.8	16.5	7.9	4.0	1.1	1.1	0.8	0.8
<i>T.hybridum</i>	EC401701	3.3	13.7	7.7	3.9	1.1	0.9	0.7	0.8
<i>T.hybridum</i>	EC425029	8.7	76.0	24.7	10.1	2.8	2.0	1.0	1.4
<i>T.hybridum</i>	EC425030	6.7	42.0	14.2	6.4	1.4	1.1	0.8	0.8
<i>T.hybridum</i>	EC425032	5.3	28.7	9.6	8.2	2.4	1.5	1.0	1.3
							Contd...		

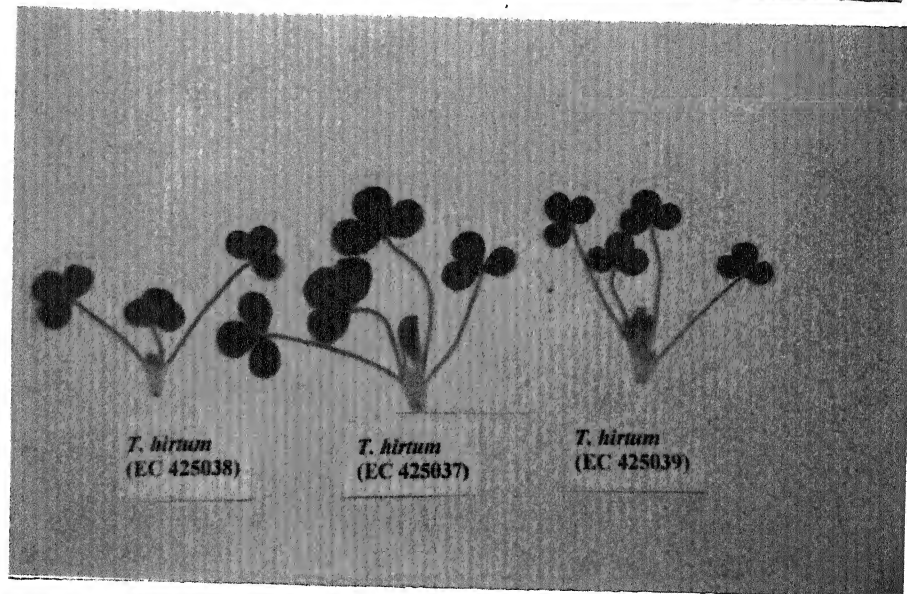
Table 4.2 (Contd.) :									
Species	Accn. No.	ch1	ch2	ch3	ch4	ch5	ch6	ch7	ch8
<i>T.subterraneum</i>	EC401718	8.7	52.3	19.7	7.8	1.3	1.5	0.8	0.7
<i>T.subterraneum</i>	EC401717	10.0	66.0	20.1	7.6	1.3	1.4	0.9	0.8
<i>T.subterraneum</i>	EC402167	7.7	70.7	16.1	6.5	1.0	1.1	0.6	0.5
<i>T.subterraneum</i>	IG 96-112	7.5	61.0	21.0	8.3	1.6	1.8	0.7	0.7
<i>T.subterraneum</i>	IG 96-113	5.5	38.5	17.4	6.6	1.2	1.5	0.7	0.6
<i>T.glomeratum</i>	EC402170	3.7	28.7	13.4	7.4	1.5	1.3	0.5	0.6
<i>T.glomeratum</i>	EC425033	1.0	9.5	10.0	6.9	1.7	1.5	0.5	0.5
<i>T.glomeratum</i>	EC401700	7.0	43.0	15.7	5.1	1.3	1.2	0.7	0.5
<i>T.glomeratum</i>	EC402170	8.0	46.0	14.7	5.0	0.8	0.7	0.6	0.3
<i>T.incarnatum</i>	EC402164	7.0	142.0	19.0	7.6	1.3	1.4	0.5	0.5
<i>T.incarnatum</i>	IG 96-111	9.7	152.0	23.8	9.0	1.7	1.7	0.5	0.4
<i>T.cherleri</i>	EC401703	11.5	113.0	13.5	3.9	0.8	0.7	0.6	0.4
<i>T.nigrescens</i>	EC425049	13.0	130.0	21.7	2.3	1.5	1.0	1.4	0.6
<i>T.nigrescens</i>	EC425047	20.0	210.0	31.3	9.3	2.3	2.2	1.0	0.6
<i>T.nigrescens</i>	EC425048	21.8	169.0	28.4	2.9	1.5	1.0	1.3	0.7
<i>T.echinatum</i>	EC425076	8.7	377.0	52.0	0.3	1.6	1.1	0.4	0.7
<i>T.echinatum</i>	EC425075	7.0	74.0	13.2	2.6	1.3	1.0	1.2	0.5
<i>T.echinatum</i>	EC425077	8.0	365.0	33.0	0.7	1.8	0.8	0.5	0.6
<i>T.echinatum</i>	EC425078	7.0	220.0	26.7	0.4	2.0	0.9	0.5	0.5
<i>T.medium</i>	EC425045	1.0	7.7	4.5	2.1	1.9	1.2	0.6	0.5
<i>T.alpestre</i>	EC425043	7.0	66.0	15.0	2.7	1.3	0.9	1.1	0.5
<i>T.alpestre</i>	EC425042	10.0	76.0	18.0	1.4	1.4	0.9	1.2	0.7
<i>T.tembense</i>	EC425064	5.0	46.0	7.0	1.1	1.0	0.5	0.5	0.3
<i>T.tembense</i>	EC425066	1.0	5.0	2.0	0.8	0.4	0.2	0.3	0.2
<i>T.tembense</i>	EC425065	1.0	15.0	5.0	1.6	1.1	0.7	0.5	0.4
<i>T.tembense</i>	EC402169	5.0	25.0	10.0	4.0	1.5	1.0	0.7	0.6
<i>T.resupinatum</i>	EC425036	18.0	400.0	70.7	4.2	2.5	1.4	0.8	0.7
<i>T.resupinatum</i>	SH 98-72	27.0	700.0	39.0	6.6	1.9	1.1	1.0	1.0
<i>T.resupinatum</i>	SH 98-73	9.3	145.0	37.9	6.4	2.4	1.4	0.9	1.1
<i>T.resupinatum</i>	SH 98-86	6.0	35.0	32.7	15.0	3.5	2.0	1.9	1.4
<i>T.resupinatum</i>	SH 98-15	10.5	268.0	45.7	5.5	2.6	1.8	1.1	1.1
<i>T.purpureum</i>	EC425069	9.7	88.3	16.7	2.4	2.6	0.9	1.6	1.6
<i>T.purpureum</i>	EC425070	11.0	317.0	39.7	2.4	3.6	1.0	2.5	1.5
<i>T.angustifolium</i>	EC425062	10.5	99.0	14.3	3.1	2.2	0.6	1.5	0.6
<i>T.angustifolium</i>	EC425061	5.5	22.5	19.5	0.5	2.5	0.2	1.2	0.7
ch1 = Branch number			ch2 = Leaf number						
ch3 = Plant height(cm)			ch4 = Petiole length (cm)						
ch5 = Leaf let length (cm)			ch6 = Leaf let breadth (cm)						
ch7 = Stipule fuse length (cm)			ch8 = Stipule free length (cm)						

Plate : 1

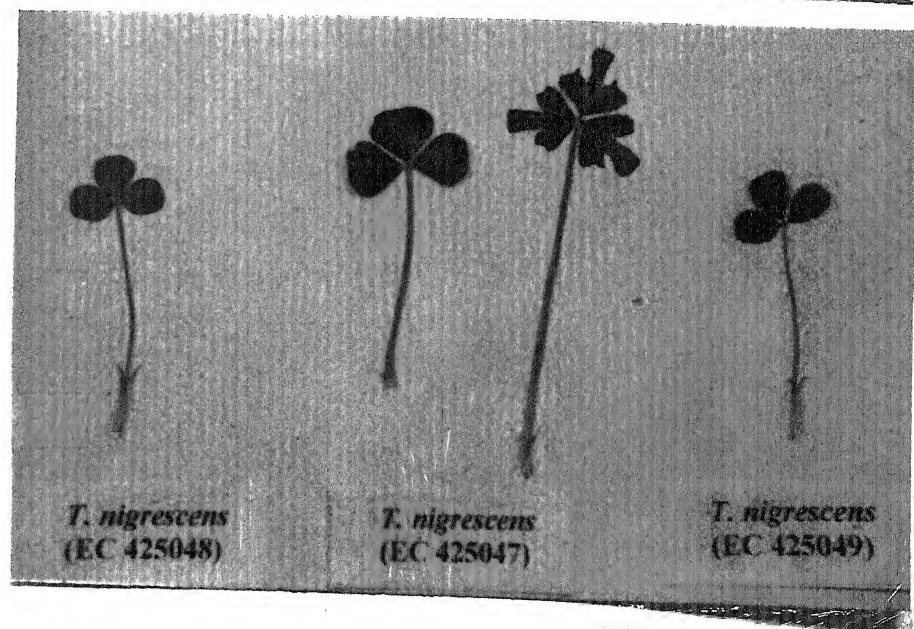
erent accessions



A



B



C

**Plate 1 : Leaf shape variation in different accessions
of *Trifolium* species.**

A. From L to R:

T. echinatum [EC 401714]

T. echinatum [EC 425076]

T. echinatum [EC 425075]

T. echinatum [EC 425077]

T. echinatum [EC 425078]

B. From L to R:

T. hirtum [EC 425038]

T. hirtum [EC 425037]

T. hirtum [EC 425039]

C. From L to R.

T. nigrescens [EC 425048]

T. nigrescens [EC 425047]

T. nigrescens [EC 425049]

41 to 105 with an average of 72. Leaves were petiolate and alternately attached to the stem. Petiole was less hairy and green in colour. Leaflets were obovate and green with entire margin. Leaflet margins and stem were marked by presence of hairs. Leaflet length and breadth ranged from 1.5 to 2.1 cm and from 1.2 to 1.5 cm respectively. Stipules had linear apex and the fused part measured 1.3 cm while free end was 0.65 cm long. No flowering was observed in local condition.

T. tembense:

The plants were annual, erect and dwarf (2 to 10 cm). The plants were agronomically poor with less branches (3 per plant) and less number of leaves per plant (5 to 46 with an average of 22.8). Stem as well as petiole were glabrous. Petiole was reddish green and short with an average length of 1.9 cm. Leaflets were elliptical, green to dark green in colour with serrate margin. Leaflet length and breadth was 1.0 cm and 0.6 cm respectively. The stipule length of fused and free portion was 0.5 cm and 0.4 cm respectively. The flower initiation was noticed in 1st week of March in EC 425065 and EC 425064 and in third week of January in EC 402169.

T. spumosum:

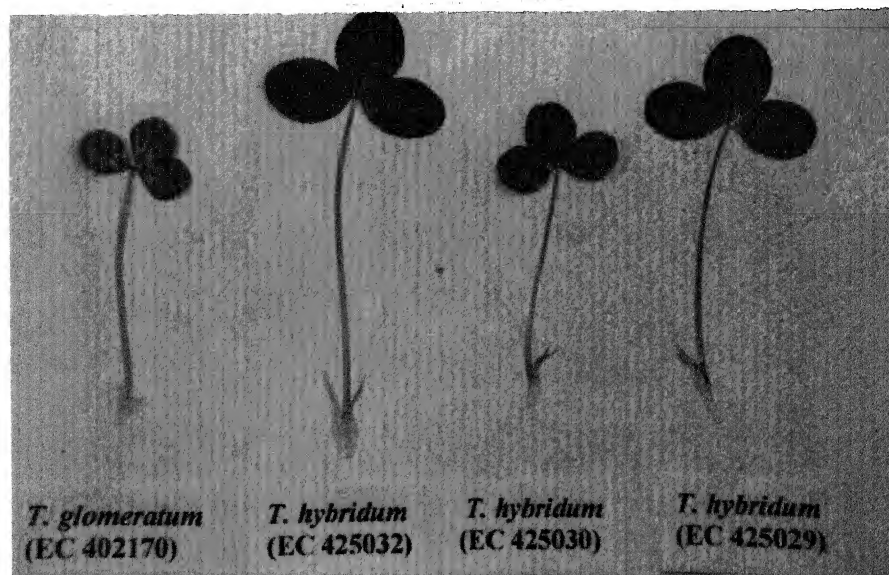
The plants of *T. spumosum* were annual, prostrate and rosette in shape with a diameter ranging from 18.0 to 22.8 cm. The primary branches ranged from 7 to 8 per plant and leaves per plant varied from 57 to 60 with an average of 59. The stem was procumbent. Leaves alternately attached to stem and were petiolate. Petiole was 8.4 cm long, glabrous and green in colour. Leaflets were rhomboid in shape, green and glabrous with serrate margin. Leaflet length and breadth ranged from 1.2 to 1.5 cm. The fused part of stipule was 0.8 cm and free ends 0.6 cm in length. The initiation of flowering was noticed in 2nd week of March.

T. lappaceum:

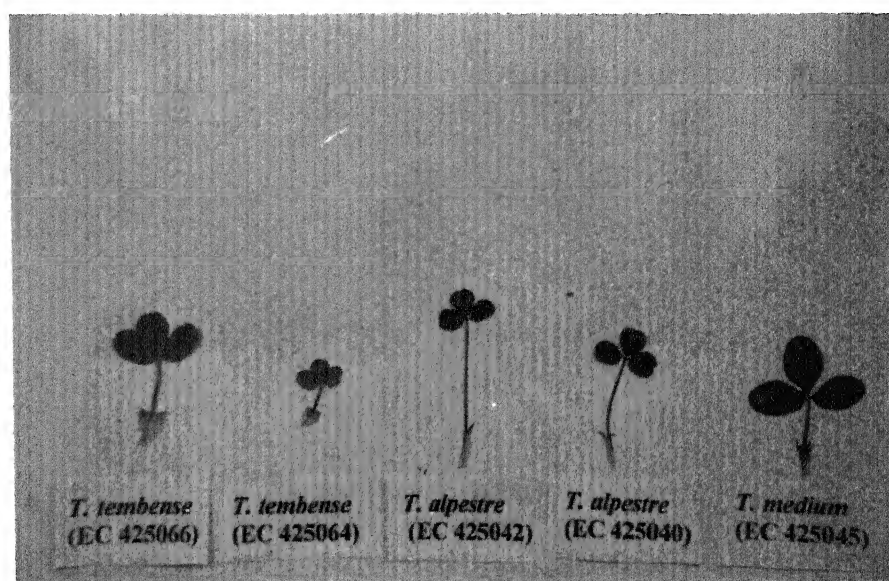
The plant were annual and erect attaining a height of 7.5 to 9.0 cm. The average number of branches per plant was 6.67 and the number of leaves per plant was 30 to 40 with an average of 35. Leaves were alternately attached to stem and were petiolate. Petiole was short to medium with an average 2.5 cm in length, glabrous and green in colour. Leaflets were obovate and dark green in colour with

Plate : 2

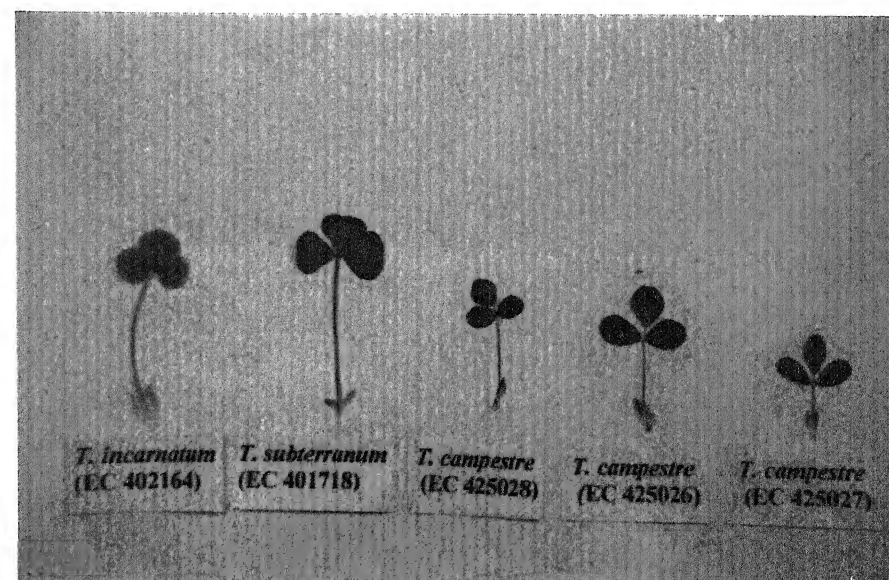
species of *Trifolium*



A



B



C

Plate 2 : Variation for leaf shape in different species of *Trifolium*.

A. From L to R:

T. glomeratum [EC 402170]

T. hybridum [EC 425032]

T. hybridum [EC 425030]

T. hybridum [EC 425029]

B. From L to R:

T. tembense [EC 425066]

T. tembense [EC 425064]

T. alpestre [EC 425042]

T. alpestre [EC 425040]

T. medium [EC 425045]

C. From L to R:

T. incarnatum [EC 402164]

T. subterraneum [EC 401718]

T. campestre [EC 425028]

T. campestre [EC 425026]

T. campestre [EC 425027]

medium hair density. The hairs were noticed at the margin of the leaflets also. Leaflet length and breadth ranged from 0.7 to 0.9 cm and 0.6 to 0.8 cm respectively. Stipules were oblong, hairy and subulate in shape and 1.2 cm long (fused part 0.8 cm and free 0.4 cm). Flowering started in 3rd week of March.

T. argutum:

The plants were annual and prostrate in nature giving a compact rosette appearance due to profuse secondary branching. Rosette diameter varied from 9.3 to 15.3 cm with an average of 13.2 cm. The average number of primary branches were 6.3 per plant and the number of leaves per plant ranged from 50 to 90 with an average of 73.3. Stem was reddish green in colour. Petiolate leaves were alternately attached to the stem. Petiole was 5.2 cm long, glabrous and reddish green. Leaflets were obovate, green, glabrous with serrate margin. Leaflet length and width range from 1.0 to 1.2 cm and 0.8 to 0.9 cm respectively. The fused part of the stipule was 0.5 to 0.6 cm long and free portion 0.4 to 0.5 cm long. The plants did not flower in Jhansi condition.

T. arvense:

Semi prostrate plants of the species were annual in nature. The plants attained 18.0 to 22 cm rosette diameter. The average number of primary branches was 6 per plant and number of leaves per plant was 50. Stem was reddish green in colour. Petiolate leaves were alternately attached to stem. Petioles were long and ranged from 7.5 to 8.0 cm with an average of 7.7 cm. Petiole was glabrous and reddish green in colour. Leaflets were obovate, green with serrate margin. Leaflet length and breadth ranged from 1.5 to 1.8 cm and 1.1 to 1.2 cm respectively. The fused part of stipules ranged from 0.7 to 1.0 cm with an average of 0.8 cm and free part ranged 0.5 to 0.7 cm, with an average of 0.57 cm. Flowering started in the 1st week of February.

T. campestre:

The plants were annual and prostrate except in EC 425026 where it was semi erect in nature. The average plant height was 23.3 cm. The average number of primary branches was 8.7 per plant and number of leaves per plant ranged from 17 to 477 with an average of 238.3. Stem was violet green in colour. Leaves alternately

attached to the stem and were petiolate. Petiole was low to medium hairy, violet green to reddish green in colour. Leaves were obovate / elliptical in shape and light green to green in colour with entire / serrate margin. Leaflet length and breadth ranged from 0.8 to 1.3 cm and 0.5 to 1.2 cm respectively. Stipules were obovate to dilated in shape and fused part and free ends reaching 0.4 cm each. The initiation of flowers was noticed in the 2nd week of March.

T. apertum:

The plants were annual, prostrate profusely branched, compact rosette in shape with a diameter ranging from 21.3 to 25.0 cm. The average number of primary branches was 11.3 per plant and number of leaves per plant ranged from 150 to 190 with a average of 167. Leaves were petiolate and alternate. Petiole was medium hairy with the average length of 7.3 cm and violet green in colour. Leaflets were obovate, dark green in colour and entire margin. Leaflet length and breadth ranged from 1.5 to 1.7 cm and 1.0 to 1.4 cm respectively. The fused part of stipule reaching from 1.1 to 1.2 cm and free ends 0.8 to 0.9 cm. The initiation of flowers was noticed in the last week of March.

T. constantinopolitanum:

The plants were annual, prostrate giving compact rosette appearance of 19 to 22 cm diameter. The plants were characterized by profuse secondary branching and large number of leaves (120 per plant). Stem was hairy. Leaves alternately attached to stem and were petiolate. Petiole had low density of hairs, were reddish green in colour and very long (7.7 cm). Leaflets were elliptical in shape and green in colour with hairy margin, 1.2 cm long and 1.0 cm broad. The fused and free portion of stipule was 0.9 cm and 0.5 cm long respectively. The initiation of flowering was noticed in 3rd week of March.

T. hybridum:

The plants of this species were perennial and semi prostrate except those of accession EC 425032 which were erect. The average number of primary branches was 5.7 per plant which were 12.6 cm long and possessed 35.3 leaves per plant. Stem was green in colour. Leaves alternately attached to the stem and were petiolate. The petiole was long and glabrous with reddish green colour but in EC 425029 and

EC 425030 the petiole colour was violet green. Leaflets were rhomboid in EC 401702 and EC 401701, obovate in EC 425029 and EC 425030 and elliptical in EC 425032. The colour of leaves was green to dark green and glabrous with serrate margin. The average leaflet length and breadth was 1.8 cm and 1.3 cm respectively. The stipule was obovate to lanceolate in shape and fused part reaching 0.9 cm and free ends 1.2 cm long. The initiation of flowering was noticed in the 1st week of April in EC 401701 and EC 401702 while in EC 425032 and EC 425029 the flower initiation was noticed in the 1st week of May.

T. subterraneum:

The species was characterized by annual and prostrate plants. Marked diversity was observed in different accessions for morphological traits. The average number of primary branches was 7.9 per plant and the number of leaves per plant ranged from 38.5 to 70.7 with an average of 57.7. Stem was reddish green in colour. Leaves were alternate with long reddish green petiole. Scanty presence of hairs on petiole was noticed in EC 402167, dense hairs in EC 401717 whereas EC 401718, IG 96-112, IG 96-113 were with medium hairs. Leaflets were ovate except in IG 96-113 where leaflets were elliptical. Leaflet length and breadth ranged from 1.0 to 1.6 and 1.1 to 1.8 cm respectively. Stipules were ovate to oblong in shape and medium in size with both fused and free portion 0.7 cm long. The initiation of flowering was noticed in 2nd week of February while in IG 96-113 the flower initiation was observed in the 1st week of March.

T. repens:

The perennial plants were spreading in nature reaching a diameter of 10.0 to 13.3 cm. The average number of primary branches was 5.7 per plant and number of leaves ranged from 23 to 35 with an average of 28.3. Stem was glabrous and green in colour. Leaves alternately attached to stem and were petiolate. Petioles were glabrous, reddish green to violet green in colour. Leaflets were rhomboid in shape, green to dark green in colour, and possessed a white 'V' shaped marker. Leaflet margin was serrate in EC 401708 and EC 400985 and entire in EC 400986. Leaflets were medium in size ranging from 1.0 to 1.3 cm length and 1.0 to 1.2 cm in breadth. Stipules were pale green in colour and small (fused portion 0.6 cm long

and free portion 0.5 cm long). The flowering initiation was noticed in the 3rd week of May.

T. glomeratum:

The annual plants of *T. glomeratum* accession EC 401700 were prostrate, EC 402170 semi prostrate and that of EC 425033 erect. The average number of primary branches per plant was 4.9 and the leaves per plant were 27.1. Petiolate leaves were green to reddish green in colour. Leaflets were obovate, light to dark green with glabrous surface. A white spot was observed in center of leaflets on midrib. The leaflets with serrate margin were 0.8 to 1.7 cm long and 0.7 to 1.5 cm wide. Stipule were ovate in shape and its fused portion was 0.6 cm long whereas free portion was 0.5 cm long. In Jhansi condition the initiation of flowering was noticed in the 2nd week of April.

T. incarnatum:

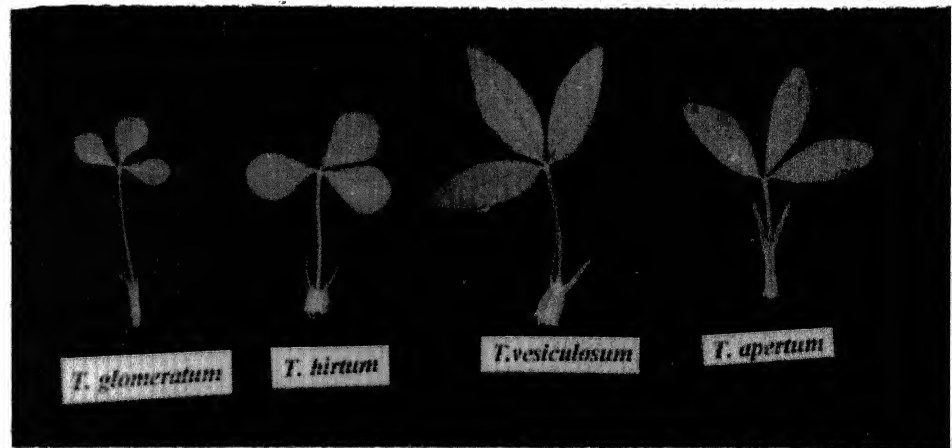
The annual plants were semi prostrate in juvenile stage and erect on maturity. The average number of primary branches per plant was 8.3 and the range of leaves per plant was from 142 to 152 with an average of 147. Leaves were petiolate and petiole was 8.3 cm long and green in colour with dense epidermal hairs in EC 402164 and glabrous in IG 96-111. Leaflets were obovate and green with dense hairs, with entire margin and 1.3 to 1.7 cm long and 1.4 to 1.7 cm wide. The stipules were small, obtuse, dentate and 0.9 cm long. The plants flowered in the 3rd week of February.

T. pratense:

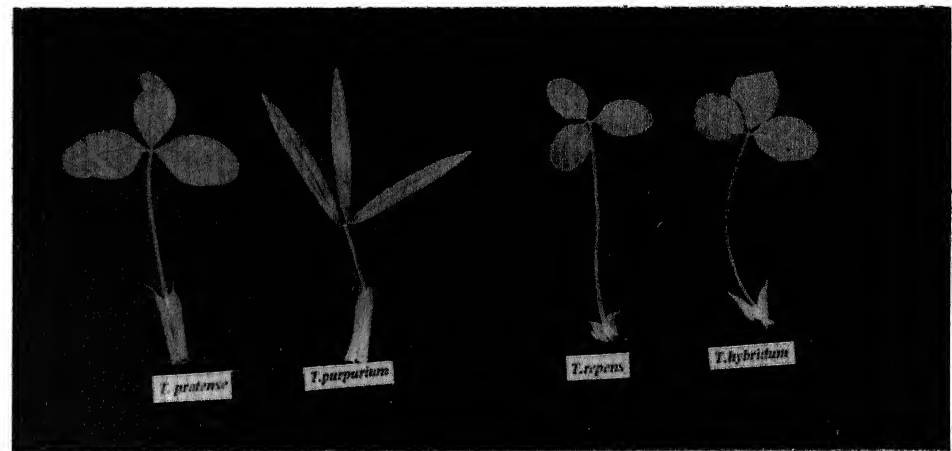
Annual and erect plants showed poor agronomic growth in Jhansi condition with less primary branches (3.1 per plant) and less number of leaves (16.2 per plant). Leaves were petiolate and green to dark green in colour. Leaflets were obovate, green to bright green with glabrous to dense hairy surface. Dense hairs on leaflets were present in EC 400735 and EC 400980. The leaflets were with entire margin and large in size (1.8 cm long and 1.5 cm wide). Stipules were triangular to ovate in shape and its fused portion was 0.7 cm long whereas free portion was 0.5 cm long. The plants flowered in the 4th week of April.

Plate : 3

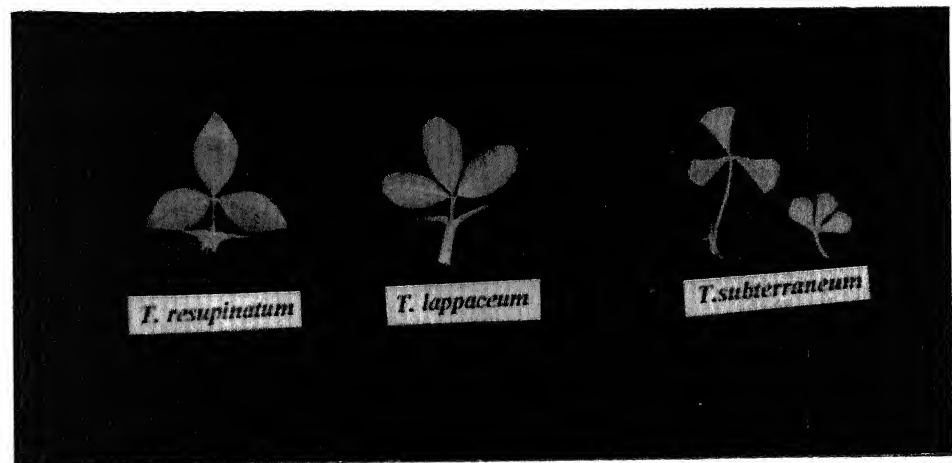
ies of *Trifolium*.



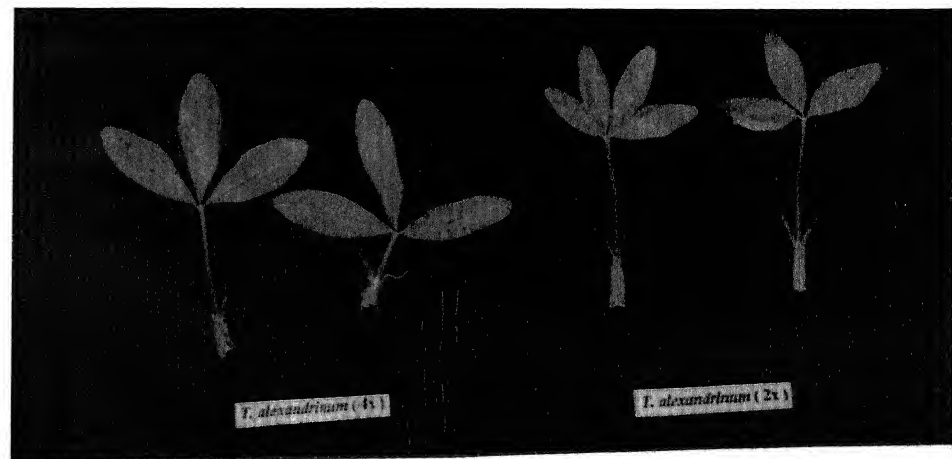
A



B



C



D

Plate 3 : Variation for leaf shape in different species of *Trifolium*.

A. From L to R:

T. glomeratum [EC 402170]

T. hirtum [EC 402153]

T. vesiculosum [EC 401716]

T. apertum [EC 401712]

B. From L to R:

T. pratense [EC 400735]

T. purpureum [EC 425070]

T. repens [EC 400985]

T. hybridum [EC 401701]

C. From L to R:

T. resupinatum [SH 98-36]

T. lappaceum [EC 402165]

T. subterraneum [EC 401718]

D. From L to R :

T. alexandrinum [JHTB 1-90-A1]

T. alexandrinum [JHB 146]

T. cherleri:

Plants of *T. cherleri* were annual and prostrate in nature. The average number of primary branches per plant was 11.5 and the leaves per plant were 113. Leaves were petiolate, dark green in colour and dense hairy. The obovate leaflets with serrate margins were 0.8 cm long and 0.7 cm wide. Stipules were linear in shape and its fused portion was longer (0.6 cm) as compared to free portion (0.4 cm). The initiation of flowering was noticed in the last week of March.

T. nigrescens:

T. nigrescens was annual in nature with runner type habit and a spread of 21.7 cm to 31.3 cm. The average number of primary branches and leaves per plant were 18.3 and 169.7 respectively. The petioles were green to violet green in colour, leaflets being obovate/ round and green / dark green with low to medium hairs. The leaflets were entire in EC 425048 and EC 425049, serrate in EC 425047 and 1.5 cm to 2.3 cm long and 1.0 cm to 2.2 cm wide. Stipules were membranous, triangular in shape and its fused portion was twice as long (1.2 cm) as the free portion (0.6 cm). The plants flowered in 4th week of March.

T. echinatum:

The plants of *T. echinatum* were annual and prostrate in nature except in EC 425078 where it was erect. The plant spread was 13.2 to 52.0 cm in diameter. The average number of primary branches per plant was 7.7 and leaves per plant 259. Petioles were green to violet green. Leaflets were obovate/ elliptical in shape and green to dark green in colour. Leaflet hairiness varied from low density to very high density in different accessions. The leaflets with entire margin were 1.7 cm long and 0.9 cm wide. Stipules were short, whose fused portion was 0.7 cm whereas free portion was 0.6 cm long. The initiation of flowering was noticed in the 1st week of March in EC 425076 and EC 425075 whereas EC 425077 and EC 401714 flowered in the 3rd week of March.

T. medium:

T. medium has been reported to be perennial but in Jhansi condition the species behaved as an annual. Growth of the prostrate plant was poor with single main branch having 7.7 leaves per plant. Petioles were violet green in colour and

glabrous. Leaflets were obovate and dark green in colour with glabrous surface. The leaflets with entire margin were 1.9 cm long and 1.2 cm wide. Stipules were lanceolate and ciliate and 1.1 cm long (0.6 cm fused and 0.5 cm free). The species did not flower in Jhansi condition.

T. alpestre:

The plants were annual and prostrate except EC 425042 which was erect in nature. The prostrate plants spread 15 to 18 cm in diameter. The average number of primary branches was 8.5 per plant and leaves per plant were 71. Petioles were violet green and low to medium hairy. Leaflets were obovate and dark green with low to medium hairs. Leaflets with entire margin were 1.3 to 1.4 cm long and 0.9 cm wide. The fused portion of stipule was 1.2 cm long whereas free portion was 0.6 cm long. The flowering initiation was noticed in the first week of April.

T. resupinatum:

The plants were annual and prostrate / semierect / erect in nature. The plants of SH 98-36, SH 98-15 were prostrate, that of SH 98-73, SH 98-86 semi-erect and SH 98-72 erect. The average number of primary branches per plant was 14.2. Plants were quite leafy having an average of 309.6 leaves per plants. Leaves were petiolate and green in colour with glabrous surface. Leaflets were obovate / elliptical and green to dark green with glabrous surface. The leaflets with serrate margin were 1.9 to 3.5 cm long and 1.1 to 1.2 cm wide. Stipules were long (total length 2.2 cm) and lanceolate in shape. The initiation of flowering was noticed in 4th week of March but two accessions *i.e.* EC 425036 and EC 401715 flowered in 4th week of April.

T. purpureum:

The plants of *T. purpureum* were annual and quite vigorous. Its side branches were decumbent whereas flowering shoot was erect attaining on an average 28.2 cm height. The average number of primary branches per plant were 10.3 and leaves per plant were 202.7. Petioles were dense hairy and green to red green in colour. Leaflets were elliptical, green in colour with long dense hairs on the surface. The leaflets with entire margins were 2.6 cm to 3.6 cm long and 0.9 to 1.0 cm wide. Stipules were also long (fused portion 2.1 cm + free portion 1.5 cm). The flower

initiation was observed in 4th week of March in EC 425070 and in the 2nd week of April in EC 425069.

T. angustifolium:

The plants of *T. angustifolium* closely resembled *T. purpureum* but were less vigorous. The species behaved as annuals and were semi erect in nature. The plants attained a height of 14.3 to 19.5 cm with the average of 16.9 cm. The average number of branches per plant was 8 and the leaves per plant were 60.8. Leaves were petiolate and alternately attached to stem. Petioles were green with medium to dense hair on surface. Leaflets were elliptical and green with glabrous to medium hair on the surface. The leaflets with entire margin were 2.4 cm long and 0.4 cm wide. Stipules were lanceolate in shape and its fused portion was 1.3 cm long whereas free portion was 0.6 cm long. The plants flowered in the 2nd week of April.

T. alexandrinum:

Some exotic lines of berseem (*T. alexandrinum*) were evaluated along with other species of the genus. Morphologically the plants were similar to other indigenous accessions. The erect plants attained an average height of 31.2 cm. The average number of primary branches per plant was 5.15 and leaves 40.7. Stem was hollow and green in colour. Leaves were alternately attached to the stem and petiolate. Petioles were green to violet green in colour with low to medium hair. Leaflets were elliptical and green in colour with glabrous to medium hairy surface. The leaflets with entire margin were 3.7 cm long and 1.5 cm wide. Stipules were lanceolate and free part subulate in shape and its fused part was 1.8 cm long whereas free portion was 1.1 cm long. The initiation of flowering was observed in the 2nd week of March.

Fifty advanced breeding lines of *T. alexandrinum* belonging to several groups viz. Exotic accessions, plants with red and pink flowers, tetraploids, leaf variants and regional collection were grown at IGFRI central experimental farm in the month of October. At the time of data recording i.e. 2nd week of March the plant height ranged from 44.5 cm to 67.5 cm with a mean value of 56.8 cm (Table 4.3, 4.4 & 4.5). The number of inter-nodes per primary branch varied from 6.6 per plant in a red flowered plant to 10.4 per branch in *Wardan* with an average of 8.7. Length of

Table 4.3. Morphological characters of different accessions of *Trifolium alexandrinum*

Genotypes	ch1	ch2	ch3	ch4	ch5	ch6	ch7
Wardan	Entire	medium	dense	25-Mar	3-Apr	WHITE	DG
JHB 94 P-22	Entire	dense	medium	20-Mar	31-Mar	WHITE	DG
JHB 94 -R-16	Entire	scant	medium	25-Mar	31-Mar	WHITE	DG
JHB 94 -R-35	Entire	medium	medium	20-Mar	31-Mar	WHITE	DG
JHB 94 -R-13	Entire	medium	dense	23-Mar	2-Apr	WHITE	MG
JHB 94 -R-25	Entire	medium	medium	20-Mar	31-Mar	WHITE	LG
JHB 94 P/T -34	Entire	medium	medium	31-Mar	7-Apr	WHITE	MG
JHB 57P3	Entire	medium	medium	23-Mar	4-Apr	WHITE	DG
JHB P17-1	Entire	scant	scant	25-Mar	5-Apr	WHITE	LG
Raj 7/13-14	Entire	medium	medium	18-Mar	28-Mar	WHITE	LG
JHB 15-27	Entire	medium	dense	22-Mar	2-Apr	WHITE	DG
JHB6/54 p/t	Entire	dense	dense	18-Mar	28-Mar	WHITE	LG
JB 92-1	Entire	medium	dense	31-Mar	3-Apr	WHITE	LG
BL 122	Entire	dense	dense	25-Mar	4-Apr	WHITE	LG
JHB 146	Entire	medium	medium	20-Mar	31-Mar	WHITE	LG
Raj 7/49-50	Entire	dense	dense	18-Mar	27-Mar	WHITE	MG
Raj 7/13-14-O	Entire	Medium	Dense	2-Apr	10-Apr	WHITE	LG
JHB 5-13/12	Entire	Medium	Medium	25-Mar	3-Apr	WHITE	LG
IL 4009	Entire	Medium	Medium	27-Mar	2-Apr	WHITE	MG
Raj 7/53-54	Entire	Dense	Dense	20-Mar	30-Mar	WHITE	MG
Raj 7/53-54- O	Entire	Medium	Medium	3-Apr	8-Apr	WHITE	LG
Raj 7/53-54 -2	Entire	Medium	Medium	2-Apr	9-Apr	WHITE	LG
JHTB 9-90N1	Serrate	Medium	Dense	31-Mar	5-Apr	W&P	DG
IL 40010-Mes	Entire	Dense	Dense	29-Mar	5-Apr	W&P	LG
JHB 94 -18/11	Entire	Medium	Scant	26-Mar	3-Apr	W&P	MG
JHB 91 P-20	Entire	Medium	Dense	15-Mar	28-Mar	W&P	DG
JHB 34/22	Entire	Dense	Dense	28-Mar	5-Apr	W&P	LG
						Contd..	

Table 4.3. Contd..							
Genotypes	ch1	ch2	ch3	ch4	ch5	ch6	ch7
Raj - Bundi - O	Entire	Medium	Medium	22-Mar	31-Mar	W&P	LG
JHB -P- 23/35	Entire	Medium	Medium	25-Mar	3-Apr	W&P&R	DG
JHTB -1-90-A1	Serrate	Medium	Medium	14-Mar	28-Mar	W&P&R	MG
JHB 94 -31	Entire	Dense	Dense	20-Mar	30-Mar	P	MG
JHB 94 -25	Entire	Medium	Medium	22-Mar	2-Apr	W&P&R	MG
IL 40014	Entire	Medium	Dense	15-Mar	30-Mar	W&P&R	MG
IL 40013	Entire	Medium	Dense	25-Mar	5-Apr	W	MG
JHB 94-P-60	Entire	Medium	Medium	25-Mar	5-Apr	W	LG
BL 144	Entire	Dense	Dense	31-Mar	5-Apr	W	LG
JHB94-56	Entire	Dense	Dense	28-Mar	5-Apr	W	DG
BL 142	Entire	Medium	Dense	15-Mar	30-Mar	W	LG
Raj 7/13-25	Entire	Dense	Dense	14-Mar	31-Mar	W&R	LG
HFB 155	Entire	Dense	Dense	10-Mar	25-Mar	W&P	MG
BL 131	Entire	Dense	Dense	18-Mar	30-Mar	W&P	LG
JHB 36/5-54	Entire	Medium	Dense	22-Mar	4-Apr	W	LG
JHB CT2 6/35	Entire	Medium	Medium	15-Mar	30-Mar	W	LG
JHB 6/54	Entire	Medium	Dense	22-Mar	3-Apr	W	LG
JHB 16/2	Entire	Medium	Dense	27-Mar	6-Apr	W	LG
HFB 155	Entire	Medium	Medium	26-Mar	5-Apr	W/P/R	LG
Wardan S-1	Entire	Medium	Dense	24-Mar	4-Apr	W	DG
Wardan S-2	Entire	Medium	Dense	25-Mar	2-Apr	W	DG
Wardan S-3	Entire	Medium	Dense	24-Mar	4-Apr	W	LG
Wardan S-4	Entire	Medium	Dense	18-Mar	30-Mar	W	LG
LG = Light Green	MG= Medium Green		DG = Dark Green				
W= White	P=Pink		R=Red				
ch1 = Leaf Margin	ch2 = Leaf hairiness	ch3 = Stipule hairiness	ch4 = Date of flowering initiation				
ch5 = Date of 50% flowering		ch6 = Flower colour	ch7 = Leaf colour				

Table 4.4. Vegetative characters and yield of different accessions of *Trifolium alexandrinum*

Genotypes	ch1	ch2	ch3	ch4	ch5	ch6	ch7
Wardan	54.0	2.3	10.4	4.3	1.0	12.1	1.4
JHB 94 P-22	51.7	5.5	9.4	3.9	1.3	11.4	2.1
JHB 94 -R-16	61.1	7.4	7.3	3.4	1.3	6.5	2.2
JHB 94 -R-35	59.5	6.8	8.7	2.9	0.8	5.0	1.9
JHB 94 -R-13	52.4	5.0	8.0	3.3	1.4	6.6	3.4
JHB 94 -R-25	53.7	4.3	6.6	3.2	1.2	7.0	2.4
JHB 94 P/T -34	66.9	5.8	8.5	4.3	1.3	9.5	2.2
JHB 57P3	61.4	4.9	9.6	3.2	1.1	8.2	1.9
JHB P17-1	67.5	5.8	8.5	3.7	1.3	10.5	2.2
Raj 7/13-14	64.0	4.8	9.6	4.0	1.3	9.0	2.1
JHB 15-27	62.4	4.6	7.4	4.0	1.3	8.8	2.4
JHB6/54 p/t	57.0	4.5	9.3	2.9	1.0	9.8	1.3
JB 92-1	55.7	3.0	9.3	3.8	1.4	8.0	1.1
BL 122	44.5	3.8	7.8	3.9	1.1	10.5	1.8
JHB 146	56.6	5.3	9.6	3.3	0.9	7.4	1.5
Raj 7/49-50	59.8	4.5	7.4	3.3	1.0	7.6	2.1
Raj 7/13-14- O	50.4	5.3	9.6	3.9	1.5	7.9	1.3
JHB 5-13/12	60.2	8.2	8.9	3.6	1.2	9.5	2.5
IL 4009	57.7	7.3	9.3	3.2	1.1	9.6	2.2
Raj 7/53-54	63.0	7.9	9.6	3.5	1.1	9.7	1.4
Raj 7/53-54- O	56.7	6.8	10.0	3.8	1.1	9.7	1.6
Raj 7/53-54 -2	56.7	6.4	10.3	3.8	1.0	8.3	1.7
JHTB 9-90 N1	60.8	9.0	9.6	3.7	1.2	9.7	1.7
IL 40010-Mes	48.5	3.4	7.8	3.6	1.6	9.1	2.7
JHB 94 -18/11	63.0	8.4	9.5	3.9	1.3	9.0	2.1
JHB 91 P-20	55.6	6.0	8.5	4.3	1.4	8.1	3.3
JHB 34/22	51.8	4.3	8.1	3.4	1.3	8.2	3.1
						Contd..	

Table 4.4 Contd..									
Genotypes	ch1	ch2	ch3	ch4	ch5	ch6	ch7		
Raj - Bundi - O	54.2	6.0	8.8	3.9	1.5	8.4	2.0		
JHB -P- 23/35	56.9	6.6	9.2	3.8	1.2	10.8	2.7		
JHTB -1-90-A1	56.2	7.2	8.1	4.0	1.2	7.6	3.0		
JHB 94 -31	58.6	6.1	8.1	3.3	1.3	10.1	2.6		
JHB 94 -25	58.9	6.8	8.5	3.8	1.3	9.1	2.6		
IL 40014	59.3	6.0	8.8	3.7	1.3	9.1	2.8		
IL 40013	53.5	5.2	7.4	4.2	1.5	11.8	2.9		
JHB 94-P-60	46.5	4.0	7.1	3.4	1.3	7.2	3.0		
BL 144	47.1	4.0	9.5	4.2	1.3	7.1	2.9		
JHB94-56	56.9	5.0	9.5	3.9	1.3	9.8	2.2		
BL 142	54.5	7.3	8.5	3.1	1.1	9.1	2.3		
Raj 7/13-25	48.8	4.3	8.5	3.6	1.3	8.9	2.7		
HFB 155	59.9	6.9	7.8	3.4	1.2	9.3	3.2		
BL 131	58.9	6.3	8.5	3.8	1.3	8.3	2.5		
JHB 36/5-54	60.9	8.1	8.5	3.3	1.3	9.3	2.0		
JHB CT2 6/35	58.0	8.8	8.2	5.1	1.7	9.4	2.4		
JHB 6/54	66.3	7.5	8.9	4.6	1.4	9.7	2.5		
JHB 16/2	49.7	3.9	9.0	4.0	1.3	9.9	2.6		
HFB 155	53.3	4.3	9.7	4.9	1.5	8.2	2.9		
Wardan S-1	58.0	8.3	8.0	3.8	1.3	10.1	3.2		
Wardan S-2	57.6	8.0	8.0	3.9	1.2	11.8	3.0		
Wardan S-3	59.7	5.0	7.5	4.6	1.4	10.4	3.3		
Wardan S-4	54.2	10.2	8.8	4.0	1.1	9.3	2.5		
Mean	56.8	5.9	8.7	3.8	1.3	9.0	2.4		
SD (+)	5.2	1.7	0.8	8.5	0.2	1.5	0.6		
ch1 = Plant height(cm)	ch2 = GFY/3m row (kg)	ch3 = No. of internodes/tiller							
ch4 = Leaflet length (cm)	ch5 = Leaflet width (cm)	ch6 = Internode length (cm)	ch7 = Petiole length (cm)						

Table 4.5. Floral and seed characters of different accessions of *T. alexandrinum*

Genotypes	ch1	ch2	ch3	ch4	ch5	ch6	ch7	ch8	ch9
Wardan	5.5	1.0	1.5	2.4	1.9	1.6	8.5	7.3	44.2
JHB 94 P-22	5.8	1.1	1.9	4.7	1.9	1.5	7.4	7.0	56.4
JHB 94 -R-16	5.5	0.9	1.7	4.9	1.9	1.4	7.8	7.3	76.4
JHB 94 -R-35	5.2	0.8	1.5	3.8	1.8	1.4	7.1	6.7	61.6
JHB 94 -R-13	5.5	0.9	1.8	5.0	1.7	1.6	7.4	7.6	51.2
JHB 94 -R-25	6.1	1.0	1.8	5.3	2.1	1.6	7.4	7.0	68.7
JHB 94 P/T -34	6.4	1.4	2.1	6.5	1.8	1.4	7.4	7.7	60.8
JHB 57P3	6.4	1.1	1.6	3.1	1.4	1.3	6.4	7.4	60.5
JHB P17-1	6.4	1.3	1.7	5.1	1.9	1.4	6.7	7.7	67.9
Raj 7/13-14	5.2	1.3	2.1	5.4	1.7	1.2	7.4	7.4	63.4
JHB 15-27	5.8	1.4	2.3	4.7	1.7	1.3	7.4	7.1	54.0
JHB6/54 p/t	5.8	1.1	1.9	3.4	2.0	1.3	8.5	7.7	74.0
JB 92-1	6.1	1.5	1.8	4.4	1.7	1.6	8.5	8.8	70.8
BL 122	5.8	1.1	1.9	5.0	1.8	1.3	8.5	8.4	81.8
JHB 146	5.8	0.9	1.8	2.5	1.8	1.2	8.5	7.7	60.5
Raj 7/49-50	4.3	1.0	1.9	4.0	2.1	1.4	8.8	7.7	53.7
Raj 7/13-14- O	5.7	1.0	2.1	4.8	1.8	1.4	8.2	8.1	66.5
JHB 5-13/12	5.7	0.8	2.0	3.2	1.7	1.3	7.2	7.6	51.5
IL 4009	4.2	0.9	1.9	3.7	2.0	1.4	7.9	8.0	64.1
Raj 7/53-54	5.7	1.0	2.0	4.1	1.7	1.1	6.8	6.8	45.1
Raj 7/53-54- O	5.7	1.0	1.8	4.0	1.7	1.4	7.1	6.9	43.2
Raj 7/53-54 -2	6.0	1.0	2.1	4.9	1.6	1.4	6.3	8.0	56.8
JHTB 9-90 N1	5.7	1.1	2.0	3.0	1.5	1.3	6.2	7.2	50.0
IL 40010-Mes	8.1	1.5	2.5	6.4	1.8	1.4	7.7	7.6	55.4
JHB 94 -18/11	6.7	1.3	2.3	6.7	1.8	1.2	7.4	7.9	65.6
JHB 91 P-20	6.7	1.3	2.5	7.0	1.3	1.3	6.7	7.9	42.9
JHB 34/22	7.4	1.3	2.4	5.7	1.4	1.4	6.5	7.8	41.8
							Contd..		

Table 4.5. Contd..									
Genotypes	ch1	ch2	ch3	ch4	ch5	ch6	ch7	ch8	ch9
Raj - Bundi - O	7.1	1.1	2.3	6.0	2.0	1.4	7.4	7.9	62.4
JHB - P- 23/35	6.1	1.3	2.2	5.6	1.9	1.4	7.7	6.9	49.0
JHTB -1-90-A1	6.7	1.5	2.7	5.1	1.7	1.6	7.4	7.3	61.4
JHB 94 -31	6.7	1.3	2.3	6.2	2.1	1.5	9.0	7.9	67.5
JHB 94 -25	6.4	0.9	1.8	4.9	1.7	1.4	8.0	8.3	50.6
IL 40014	6.4	1.2	2.2	6.3	1.8	1.4	9.3	7.6	57.9
IL 40013	7.1	1.3	2.4	7.4	2.2	1.4	9.9	7.9	64.6
JHB 94-P-60	6.1	1.0	2.0	7.2	1.8	1.4	8.3	7.6	60.8
BL 144	7.7	1.2	2.5	4.5	2.2	1.7	7.7	7.9	57.6
JHB94-56	7.1	1.2	2.3	5.5	1.8	1.4	6.7	7.2	59.8
BL 142	7.1	1.1	2.1	5.0	1.9	2.0	8.6	7.6	66.9
Raj 7/13-25	6.7	1.2	2.3	5.3	1.8	1.3	8.3	7.3	56.3
HFB 155	7.1	1.2	2.3	4.8	1.6	1.2	7.7	6.9	29.8
BL 131	5.7	1.1	2.3	4.3	1.7	1.2	7.7	6.9	47.4
JHB 36/5-54	5.7	0.9	1.7	3.5	1.3	1.3	7.4	7.9	43.8
JHB CT2 6/35	7.0	1.5	2.1	6.1	1.8	1.4	8.0	7.4	68.3
JHB 6/54	7.7	1.4	2.4	6.3	2.3	1.7	10.2	7.4	69.4
JHB 16/2	6.1	1.0	2.2	4.3	1.8	1.3	8.8	7.9	59.1
HFB 155	6.5	0.9	1.7	5.4	1.8	1.5	8.8	7.3	68.9
Wardan S-1	5.4	1.0	1.9	4.7	1.7	1.2	7.8	7.3	44.8
Wardan S-2	7.2	1.0	2.1	5.9	1.7	1.4	7.8	7.6	53.5
Wardan S-3	6.8	1.0	1.7	4.3	1.9	1.5	6.3	6.7	63.6
Wardan S-4	5.0	0.8	1.7	5.0	1.9	1.4	8.8	6.9	65.2
Mean	6.22	1.12	2.04	4.95	1.79	1.42	7.78	7.52	58.35
SD (+)	0.82	0.19	0.29	1.17	0.22	0.14	0.91	0.46	10.27
ch1 = Stipule width (mm)		ch2 = Stipule fused length (cm)		ch3 = (Stipule total length (cm)					
ch4 = Peduncle length (cm)		ch5 = Inflorescence length (cm)		ch6 = Inflorescence width (cm)					
ch7 = No. of Whorls/ inflorescence		ch8= No. of flowers in first whorl		ch9= Seeds/inflorescence					

inter node ranged from 5.0 cm to 12.1 cm with a mean value of 9.0 cm. The leaflets were lanceolate in shape and light to dark green in colour. The leaflet length and breadth ranged from 2.9 cm to 5.1 cm and 0.8 cm to 1.7 cm respectively. Leaflet margin was entire in all 48 diploid lines and serrate in 2 tetraploid lines. Medium to dense hair intensity was noticed on leaflet surface. The fused portion of stipule ranged from 0.8 cm to 1.5 cm with a mean value of 1.1 cm whereas the total length of stipules ranged from 1.47 to 2.68 cm with an average of 2.0 cm. Stipule was 4.2 to 7.7 mm broad with a mean value of 6.2 mm and were medium to dense hairy. The initiation of flowering was noticed from 10th March to 3rd April among different lines and all the lines showed 50% flowering between 27th March to 10th April. The flower colour varied from white, pink or red in different accessions. Peduncle length ranged from 2.4 to 7.4 cm with a mean value of 5.0 cm. Mature inflorescence length varied from 1.3 to 2.3 cm with an average of 1.8 cm and mature inflorescence diameter ranged from 1.1 cm to 2.0 cm with a mean value of 1.4 cm. The number of flower whorls per inflorescence range from 6.2 to 10.2 with a mean value of 7.8 and the number of flower in 1st whorl ranged from 6.7 to 8.8 with an average of 7.5. The seeds per inflorescence ranged from 29.8 to 81.8 with a mean value of 58.4.

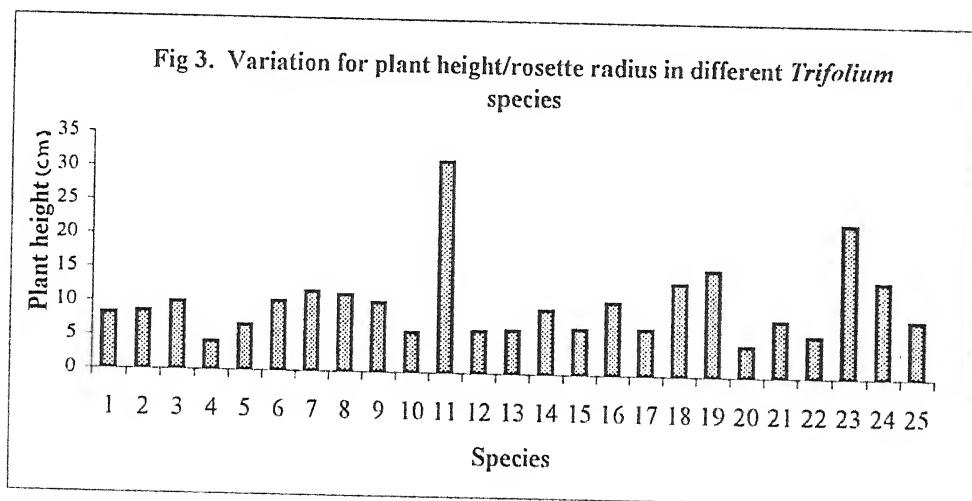
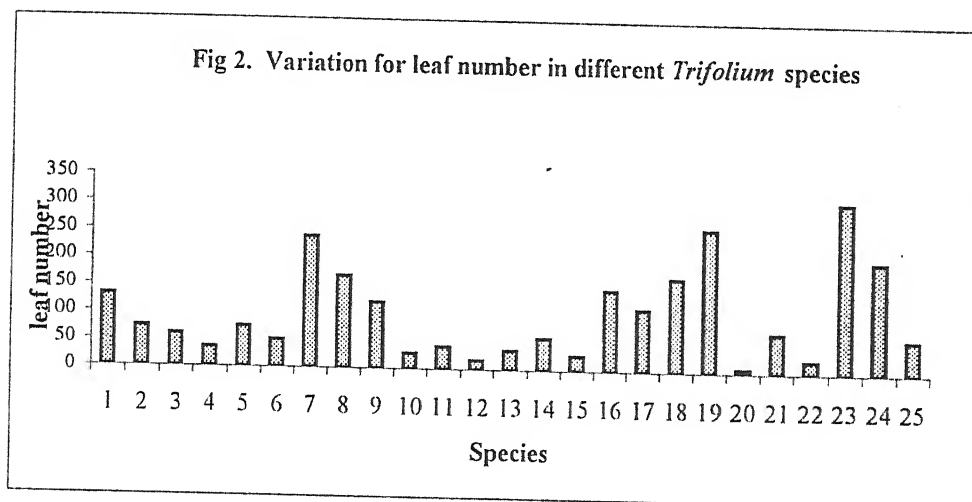
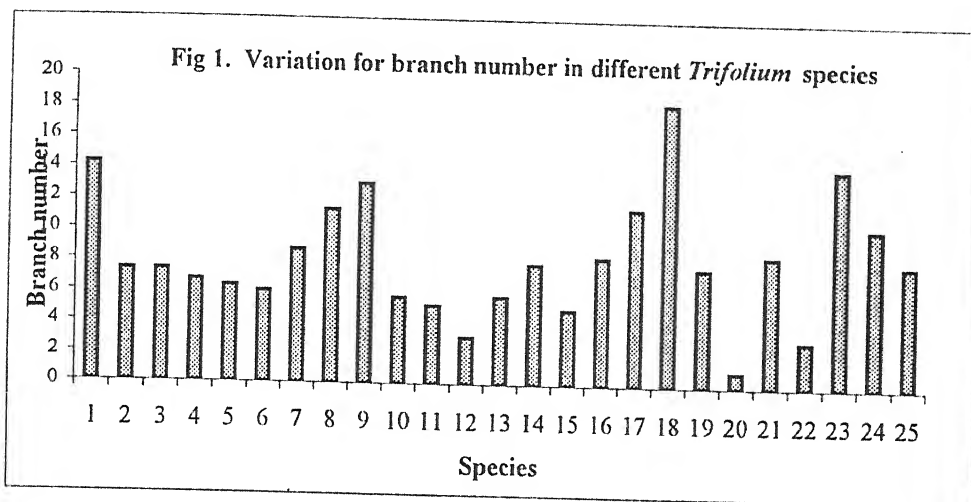
4.1.2. Interspecies variation for morphological traits

Wide diversity for various morphological characters was noticed in 25 *Trifolium* species (Table 4.6, Fig. 1 to 9). Considerable variation for branch number was observed among different *Trifolium* species. Number of primary branches per plant ranged from 1 to 18.3. Maximum 18.3 branches per plant was recorded in *T. nigrescens* followed with 14.2 branches each in *T. hirtum* and *T. resupinatum*. Single branch per plant was noticed in *T. medium* followed with 3 in *T. tembense* and 3.1 per plant in *T. pratense*. Most of the species possessed 5 to 8 branches per plant.

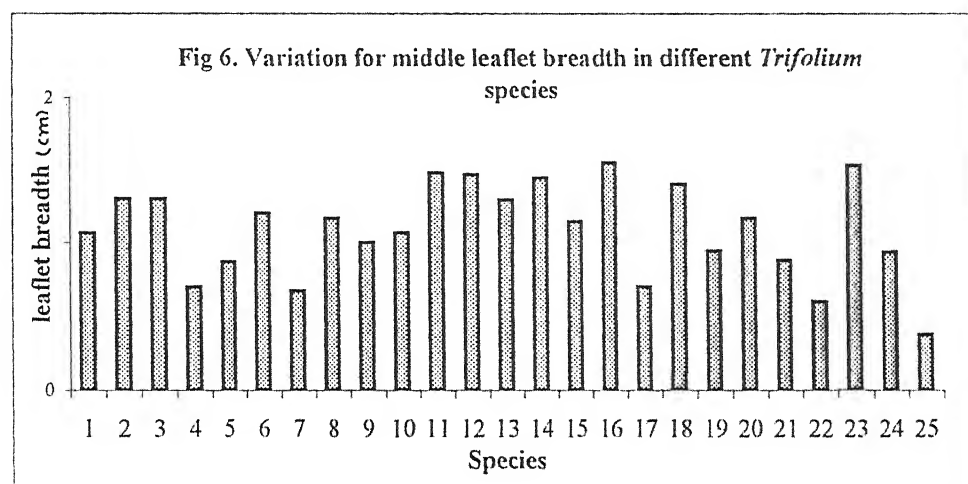
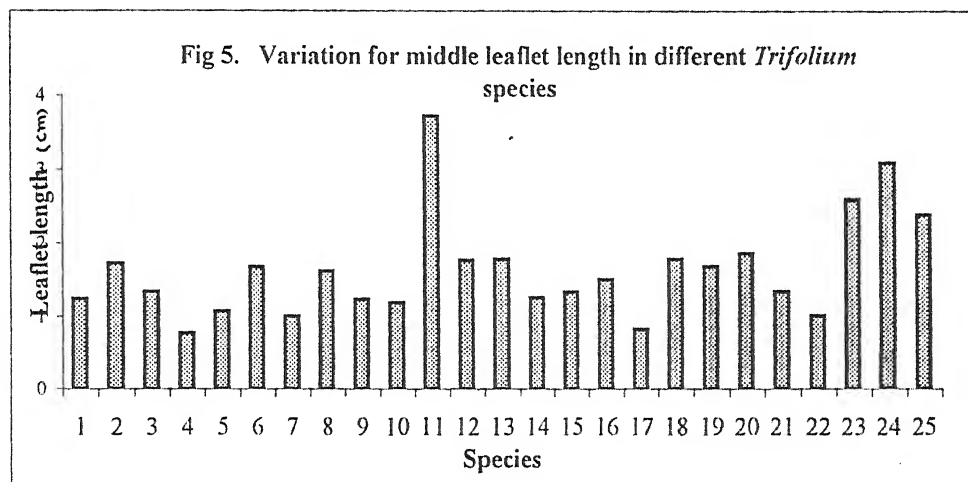
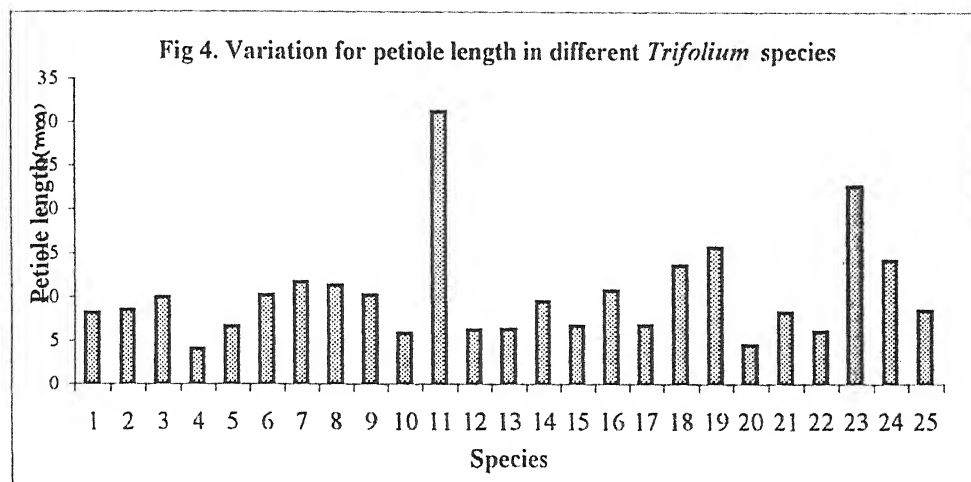
Leafiness is an important forage trait and high degree of variation for this trait was recorded among different species. Maximum 309.6 leaves were present in prostrate line of *T. resupinatum* followed with 259 in *T. echinatum*. Minimum number of leaves i.e. 7.7 were observed in *T. medium* followed with 16.2 in *T. pratense*. Around 72 leaves per plant were observed in *T. diffusum*, *T. argutum* and *T. alpestre*.

Table 4.6 : Range and mean of various morphological characters of different Trifolium species (based on table 4.2)											
Species	ch1	ch2	ch3	ch4	ch5	ch6	ch7	ch8	ch9		
<i>T.hirtum</i>	Mean	14.20	130.00	16.30	5.03	1.24	1.07	0.83			
	Range	(10-21.3)	(95-183)	(15.3-17.7)	(4.4-6.1)	(1.1-1.3)	(1.0-1.1)	(0.6-1.0)			
<i>T.diffusum</i>	Mean	7.33	72.00	17.03	6.13	1.72	1.30	1.30			
	Range	(6.2-8.9)	(41-105)	(13.8-19.7)	(4.3-8.6)	(1.5-2.1)	(1.2-1.5)	(1.1-1.4)			
<i>T.spumosum</i>	Mean	7.33	59.00	19.83	8.40	1.33	1.30	0.77			
	Range	(7-8)	(57-60)	(18-22.8)	(7.67-9.33)	(1.2-1.5)	(1.2-1.5)	(0.6-0.87)			
<i>T.lappaceum</i>	Mean	6.67	35.00	8.06	2.53	0.77	0.70	0.77			
	Range	(6-7)	(30-40)	(7.5-9.0)	(2.3-2.9)	(0.7-0.9)	(0.6-0.77)	(0.6-0.9)			
<i>T.argutum</i>	Mean	6.33	73.30	13.21	5.20	1.07	0.87	0.53			
	Range	(5.0-7.0)	(50-90)	(9.3-15.3)	(4.7-5.7)	(0.97-1.17)	(0.8-0.93)	(0.5-0.6)			
<i>T.arvense</i>	Mean	6.00	50.00	20.30	7.70	1.67	1.20	0.80			
	Range	(4.2-7.2)	(45-59)	(18.0-22.0)	(7.5-8.0)	(1.5-1.8)	(1.1-1.25)	(0.7-1.0)			
<i>T.campestre</i>	Mean	8.71	238.25	23.30	1.13	1.00	0.67	0.41			
	Range	(1-18)	(17-477)	(8.8-39)	(0.5-1.9)	(0.8-1.3)	(0.5-0.8)	((0.3-0.6)			
<i>T.apertum</i>	Mean	11.3	167.0	22.5	7.27	1.61	1.17	1.17			
	Range	(10.0-12.0)	(150-190)	(21.3-25.0)	(7.0-7.5)	(1.47-1.7)	(0.97-1.4)	(1.1-1.23)			
<i>T.constant-</i>	Mean	13.00	120.00	20.30	7.70	1.23	1.00	0.90			
<i>inopolitanum</i>	Range	(12-15)	(108-138)	(19-22)	(7.5-8.0)	(1.2-1.3)	(0.8-1.3)	(0.8-1.0)			
<i>T.repens</i>	Mean	5.667	28.333	11.667	4.933	1.180	1.067	0.600			
	Range	(5-7)	(23-35)	(10-13.3)	(4.2-5.7)	(1.0-1.3)	(1.0-1.2)	(0.6-1.3)			
<i>T.alexandrinum</i>	Mean	5.15	40.67	31.20	5.02	3.72	1.48	1.81			
	Range	(3.3-6.7)	(29-76.3)	(5-50.7)	(2.4-6.7)	(2.4-4.9)	(1.1-1.8)	(1.1-2.1)			
<i>T.pratense</i>	Mean	3.1	16.2	12.4	5.7	1.8	1.5	0.7			
	Range	(1.0-4.3)	(8.5-26)	(7-21.5)	(3.6-7.9)	(1.2-2.7)	(1.1-2.0)	(0.4-1.0)			
<i>T.hybridum</i>	Mean	5.7	35.3	12.6	6.4	1.8	1.3	0.9			
	Range	(3.3-8.7)	(13.7-76)	(7.7-24.7)	(3.9-10.1)	(1.1-2.8)	(0.9-2.0)	(0.7-1.0)			
											Contd...

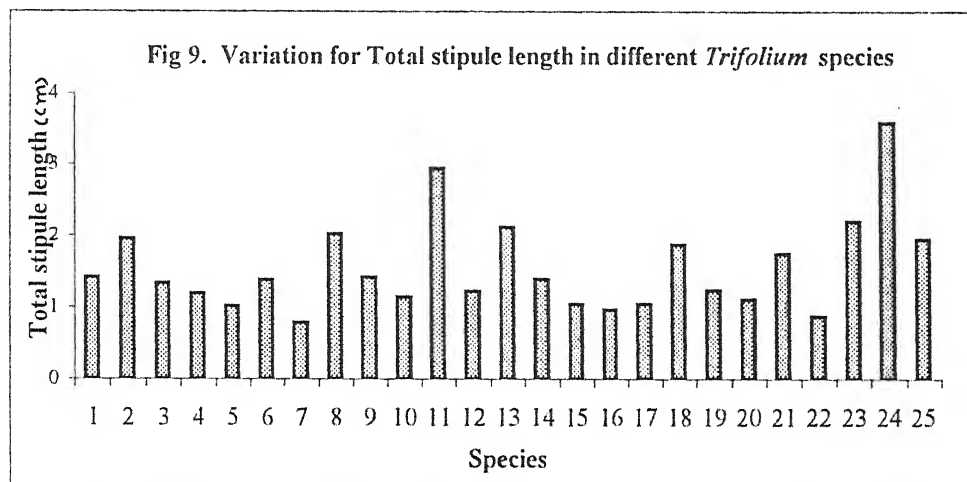
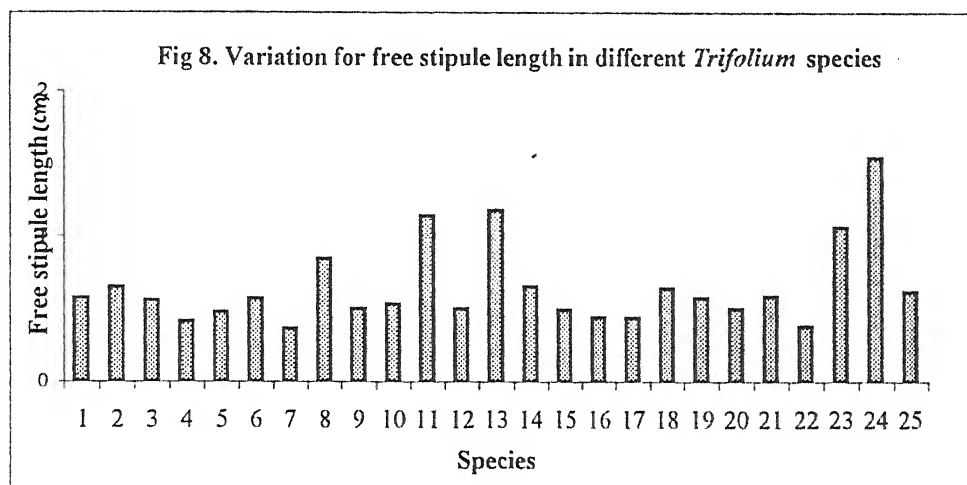
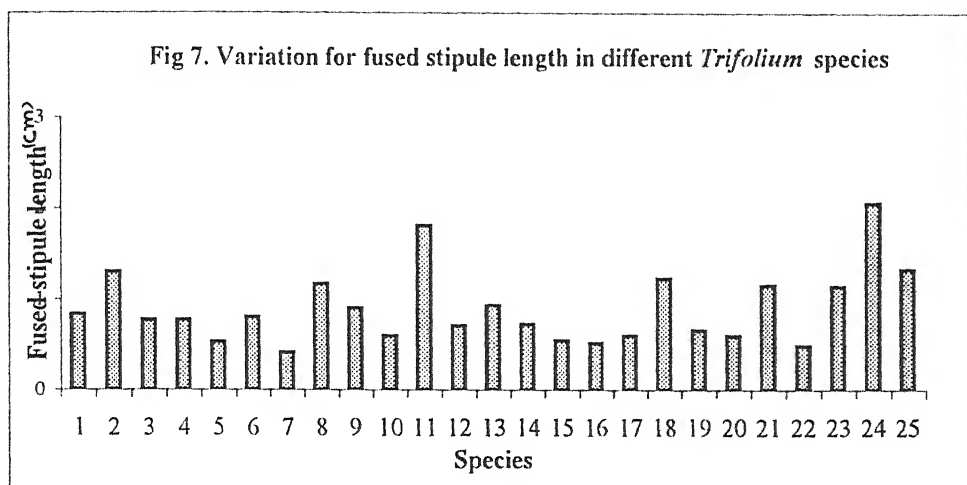
Table 4.6 (Contd..)											
Species	ch1	ch2	ch3	ch4	ch5	ch6	ch7	ch8	ch9		
<i>T. subterraneum</i>	Mean	7.9	57.7	18.9	7.4	1.3	1.4	0.7	1.4		
	Range	(5.5-10.0)	(38.5-70.7)	(16.1-21)	(6.5-8.3)	(1.0-1.6)	(1.1-1.8)	(0.6-0.9)	(0.5-0.8)		
<i>T. glomeratum</i>	Mean	4.92	27.07	13.45	6.08	1.33	1.14	0.55	0.49	1.0	
	Range	(1.0-8.0)	(9.5-46)	(10-15.7)	(5.0-7.4)	(0.8-1.7)	(0.7-1.5)	(0.5-0.7)	(0.3-0.6)		
<i>T. incarnatum</i>	Mean	8.34	147.00	21.39	8.28	1.49	1.55	0.52	0.44	0.96	
	Range	(7.0-9.7)	(142-152)	(19-23.8)	(7.6-9.0)	(1.3-1.7)	(1.4-1.7)	(0.5-0.5)	(0.4-0.5)		
<i>T. cherleri</i>	Mean	11.50	113.00	13.50	3.90	0.82	0.70	0.60	0.44	1.04	
	Range	(10-13)	(105-120)	(13.0-14.0)	(3.6-4.17)	(0.8-0.83)	(0.6-0.8)	(0.5-0.8)	(0.3-0.5)		
<i>T. nigrescens</i>	Mean	18.27	169.67	27.12	4.82	1.77	1.40	1.22	0.64	1.86	
	Range	(13.0-21.8)	(130-210)	(21.7-31.3)	(2.3-9.3)	(1.5-2.3)	(1.0-2.2)	(1.0-1.4)	(0.6-0.7)		
<i>T. echinatum</i>	Mean	7.67	259.00	31.23	1.00	1.67	0.94	0.66	0.57	1.23	
	Range	(7.0-8.7)	(74-377)	(13.2-52.0)	(0.3-2.6)	(1.3-2.0)	(0.8-1.1)	(0.4-1.2)	(0.5-0.7)		
<i>T. medium</i>	Mean	1.00	7.67	4.50	2.07	1.86	1.17	0.60	0.53	1.13	
	Range	(0.7-1.5)	(6.0-10.0)	(2.1-7.3)	(1.5-2.6)	(1.83-2.00)	(0.7-1.6)	(0.5-0.7)	(0.5-0.6)		
<i>T. alpestre</i>	Mean	8.50	71.00	16.50	2.05	1.33	0.88	1.15	0.59	1.74	
	Range	(7.0-10.0)	(66-76)	(15-18)	(1.4-2.7)	(1.3-1.4)	(0.9-0.9)	(1.1-2.0)	(0.5-0.7)		
<i>T. tembense</i>	Mean	3.00	22.75	6.00	1.88	1.00	0.60	0.49	0.38	0.87	
	Range	(1.0-5.0)	(5-46)	(2.0-10.0)	(0.8-4.0)	(0.4-1.5)	(0.2-1.0)	(0.3-0.7)	(0.2-0.6)		
<i>T. resupinatum</i>	Mean	14.17	309.60	45.20	7.55	2.59	1.53	1.14	1.06	2.19	
	Range	(6.0-27.0)	(35-700)	(32.7-70.7)	(4.2-15.0)	(1.9-3.5)	(1.1-2.0)	(0.8-1.9)	(0.7-1.4)		
<i>T. purpureum</i>	Mean	10.34	202.65	28.17	2.37	3.08	0.93	2.05	1.54	3.59	
	Range	(9.7-11.0)	(88.3-317)	(16.7-39.7)	(2.4-2.4)	(2.6-3.6)	(0.9-1.0)	(1.6-2.5)	(1.5-1.6)		
<i>T. angustifolium</i>	Mean	8.00	60.75	16.88	1.80	2.38	0.38	1.33	0.62	1.94	
	Range	(5.5-10.5)	(22.5-99)	(14.3-19.5)	(0.5-3.1)	(2.2-2.5)	0.2-0.6)	(1.2-1.5)	(0.6-0.7)		
ch1 = Branch number	ch2 = Leaf number	ch3 = Plant height	ch4 = Stipule free length	ch5 = Petiole length	ch6 = Leaflet length	ch7 = Total stipule length	ch8 = Leaflet length	ch9 = Total stipule length			
ch6 = Leaflet breadth	ch7 = Stipule fuse length	ch8 = Stipule height	ch9 = Stipule free length	ch10 = Stipule free length	ch11 = Stipule free length	ch12 = Stipule free length	ch13 = Stipule free length	ch14 = Stipule free length	ch15 = Stipule free length	ch16 = Stipule free length	ch17 = Stipule free length



1. *T. hirtum*, 2. *T. diffusum*, 3. *T. spumosum*, 4. *T. lappaceum*, 5. *T. argutum*, 6. *T. arvense*, 7. *T. campestre*, 8. *T. apertum*, 9. *T. constantinopolitanum*, 10. *T. repens*, 11. *T. alexandrinum*, 12. *T. pratense*, 13. *T. hybridum*, 14. *T. subterraneum*, 15. *T. glomeratum*, 16. *T. incarnatum*, 17. *T. cherleri*, 18. *T. nigrescens*, 19. *T. echinatum*, 20. *T. medium*, 21. *T. alpestre*, 22. *T. tembense*, 23. *T. resupinatum*, 24. *T. purpureum*, 25. *T. angustifolium*



1. *T. hirtum*, 2. *T. diffusum*, 3. *T. spumosum*, 4. *T. lappaceum*, 5. *T. argutum*, 6. *T. arvense*, 7. *T. campestre*, 8. *T. apertum*, 9. *T. constantinopolitanum*, 10. *T. repens*, 11. *T. alexandrinum*, 12. *T. pratense*, 13. *T. hybridum*, 14. *T. subterraneum*, 15. *T. glomeratum*, 16. *T. incarnatum*, 17. *T. cherleri*, 18. *T. nigrescens*, 19. *T. echinatum*, 20. *T. medium*, 21. *T. alpestre*, 22. *T. temense*, 23. *T. resupinatum*, 24. *T. purpureum*, 25. *T. angustifolium*



1. *T. hirtum*, 2. *T. diffusum*, 3. *T. spumosum*, 4. *T. lappaceum*, 5. *T. argutum*, 6. *T. arvense*, 7. *T. campestre*, 8. *T. apertum*, 9. *T. constantinopolitanum*, 10. *T. repens*, 11. *T. alexandrinum*, 12. *T. pratense*, 13. *T. hybridum*, 14. *T. subterraneum*, 15. *T. glomeratum*, 16. *T. incarnatum*, 17. *T. cherleri*, 18. *T. nigrescens*, 19. *T. echinatum*, 20. *T. medium*, 21. *T. alpestre*, 22. *T. tembense*, 23. *T. resupinatum*, 24. *T. purpureum*, 25. *T. angustifolium*

Plate 4 : Branching pattern in different *Trifolium* species.

A. *T. repens* [EC 400985]

B. *T. echinatum* [EC 425078]

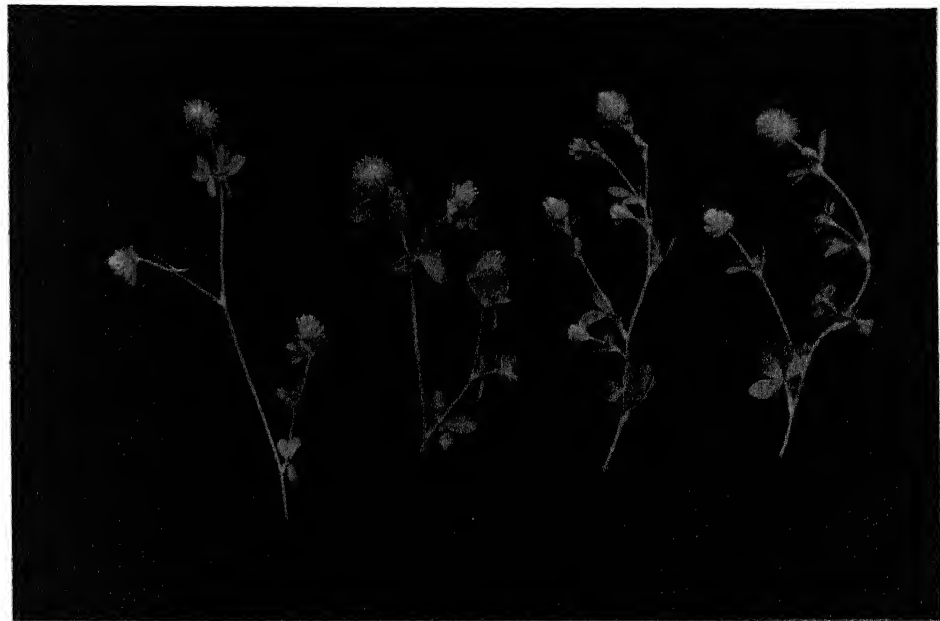
C. *T. lappaceum* [EC 402165]

Plate : 4

lium species.



A



B



C

Plate 5 : Branching pattern in different species of *Trifolium*

A. *T. pratense* [EC 400979]

B. *T. alexandrinum* [JHB 146]

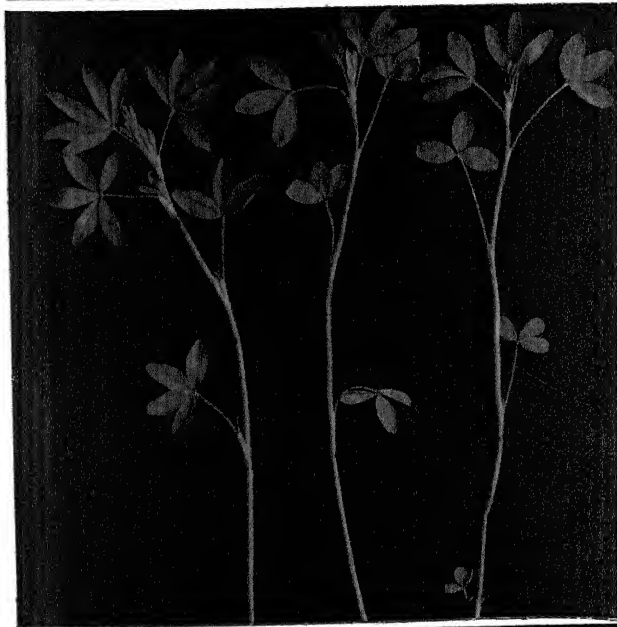
C. *T. apertum* [EC 401712]

Plate : 5

ifolium



A



B



C

Plate 6 : Flowering twig of different *Trifolium* species.

From L to R:

- A. *T. vesiculosum* [EC 401716]
- B. *T. hybridum* [EC 425030]
- C. *T. constantinopolitanum* [EC 401713]
- D. *T. resupinatum* [SH 98-15]



Plate : 6

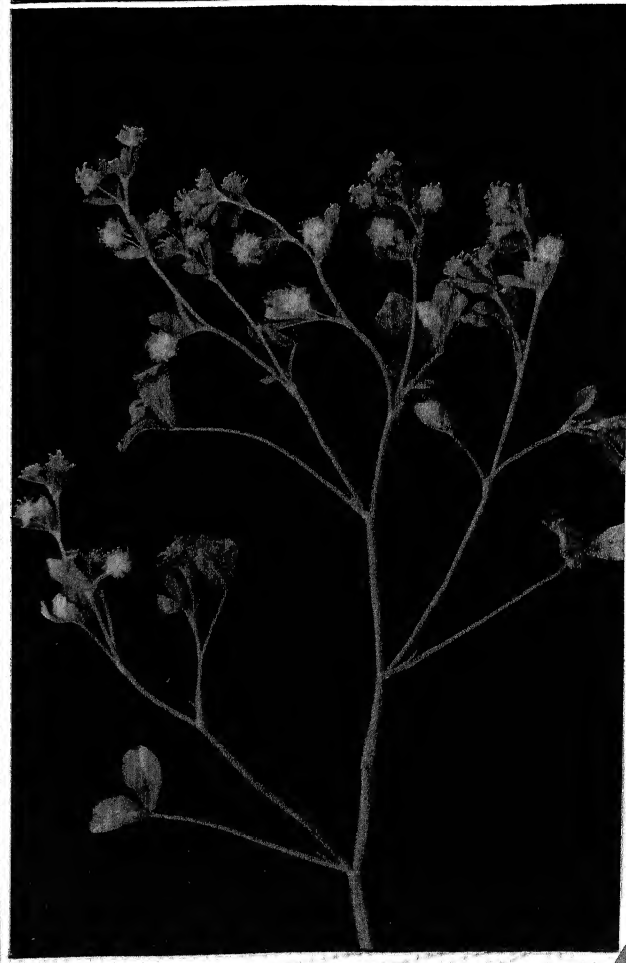
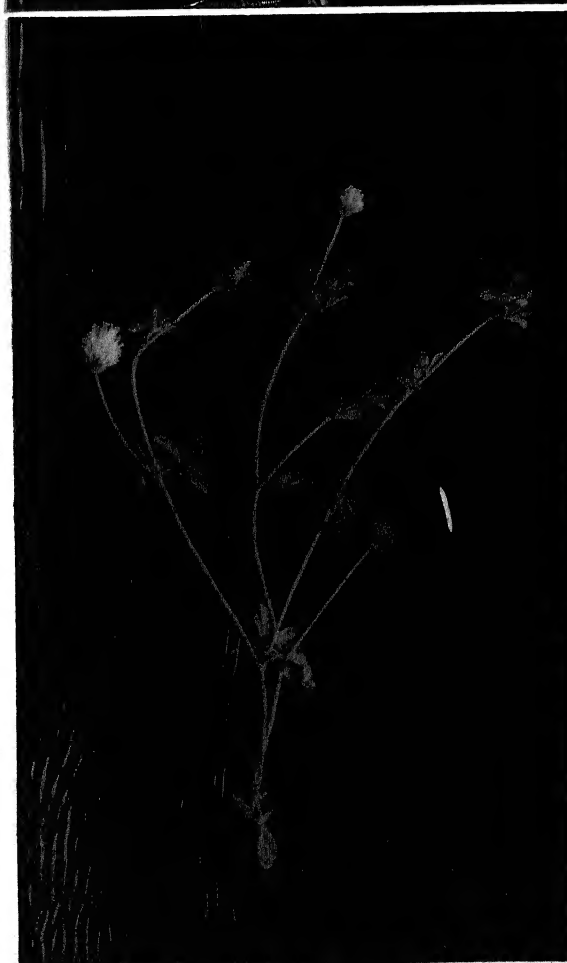
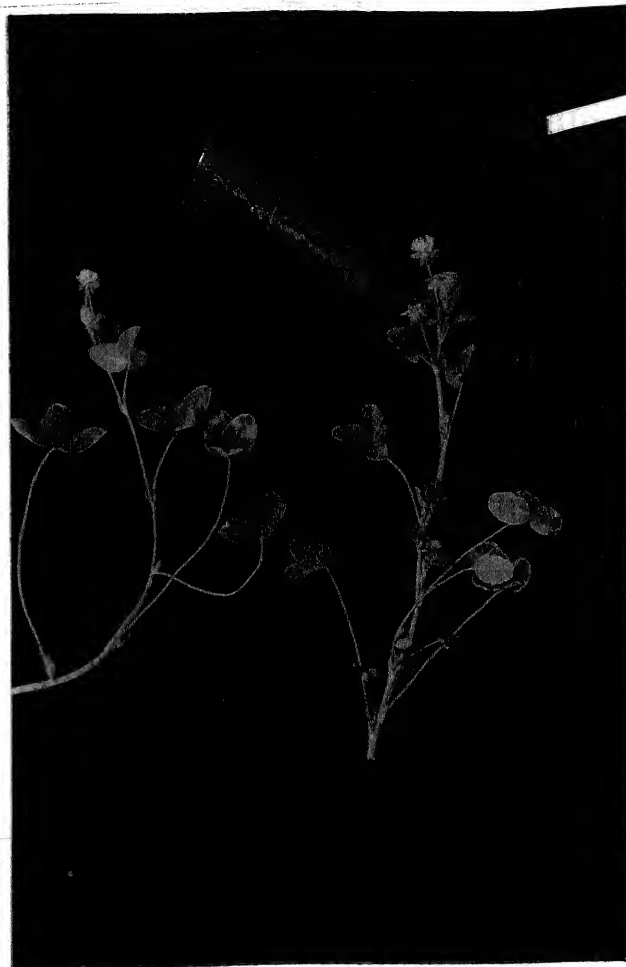


Plate 7 : Flowering twig of different species of *Trifolium*.

From L to R.

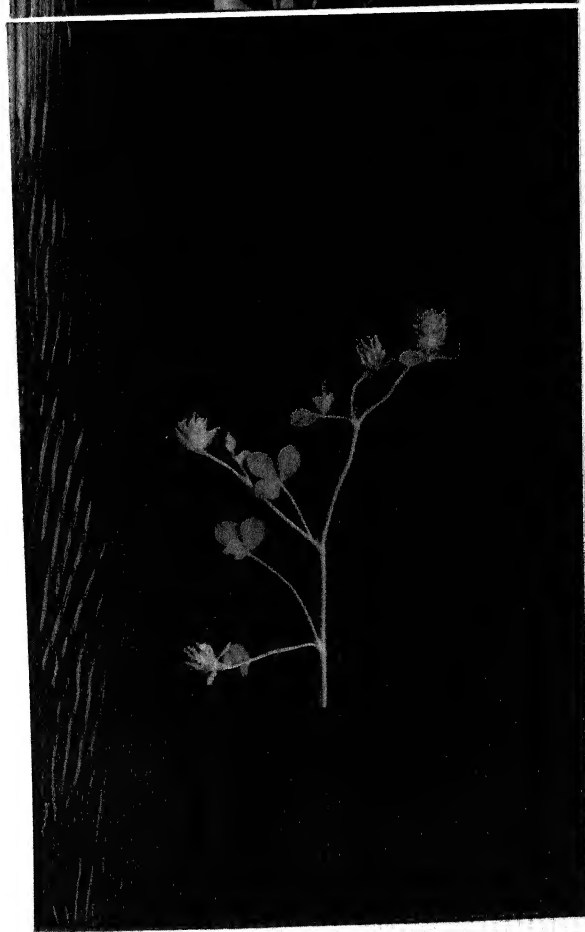
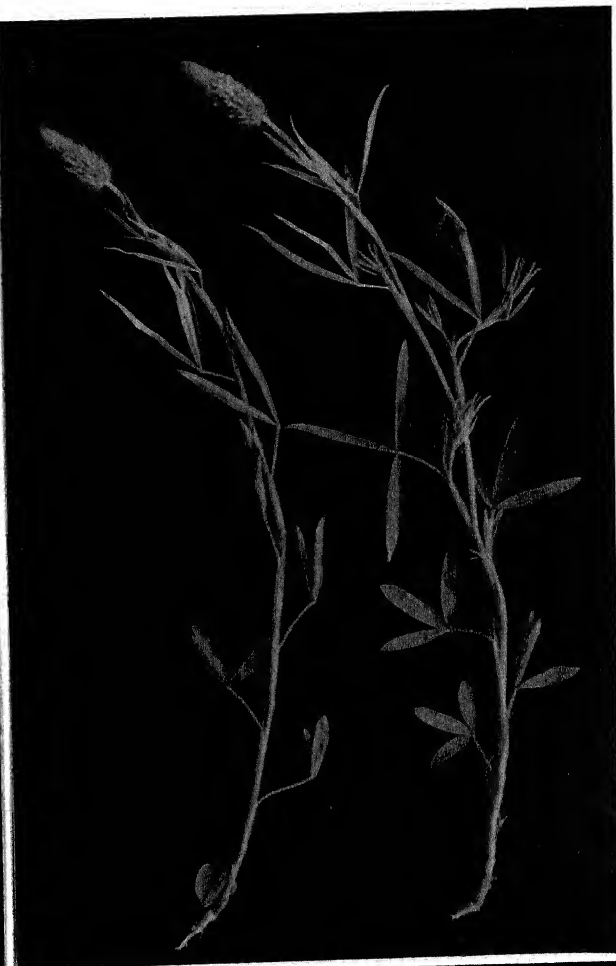
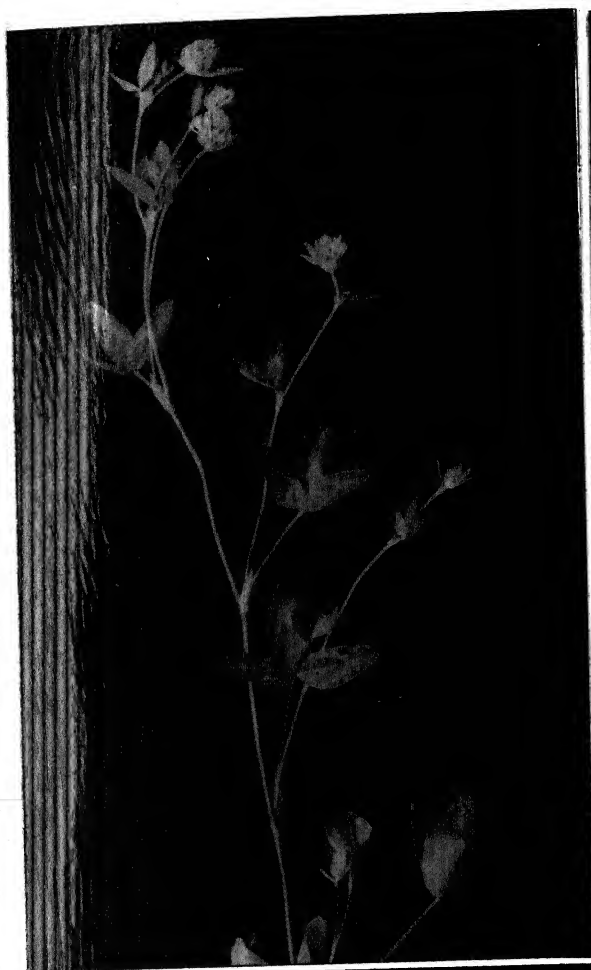
A. *T. pratense* [EC 400735]

B. *T. purpureum* [EC 425070]

C. *T. hirtum* [EC 425037]

D. *T. alexandrinum* [JHB 146]

Plate : 7



**Plate 8 : Variation for inflorescence shape and size in
different *Trifolium* species.**

A. From L to R.

T. alexandrinum [JHB 146]

T. vesiculosum [EC 401716]

T. resupinatum [SH 98-36]

T. echinatum [EC 425078]

T. campestre [EC 425028]

B. From L to R:

T. repens [EC 400985]

T. hirtum [EC 402153]

T. purpureum [EC 425070]

T. nigrescens [EC 425047]

T. pratense [EC 400735]

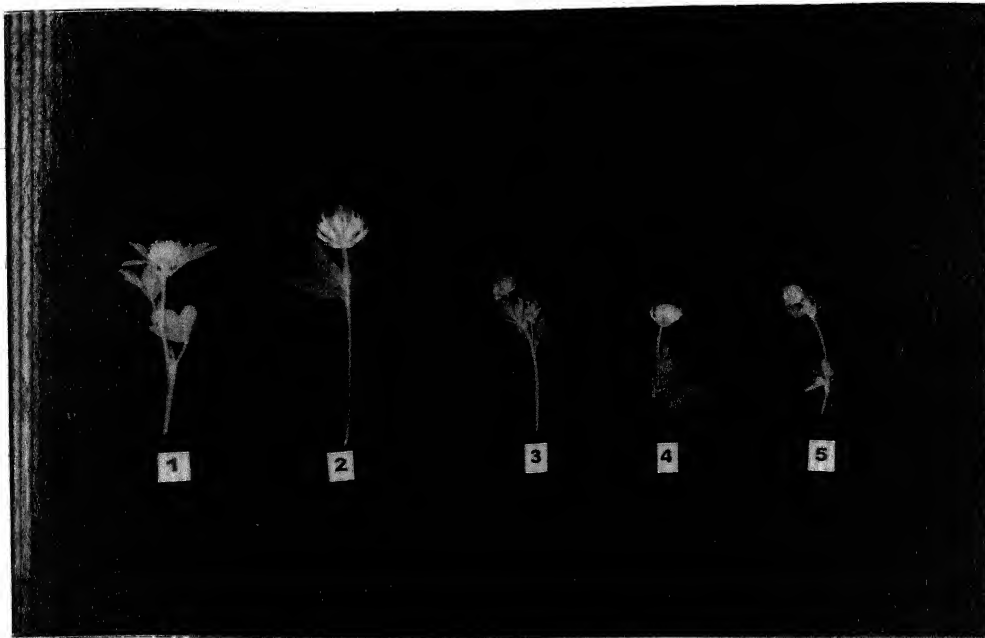
C. From L to R:

T. resupinatum [SH 98-36]

T. hirtum [EC 425037]

T. campestre [EC 425028]

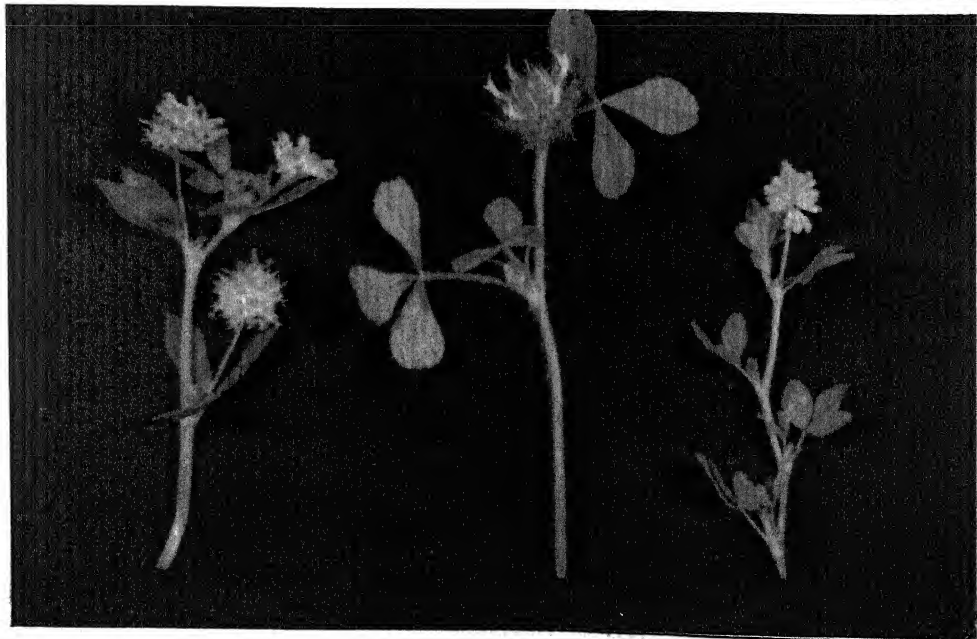
Plate : 8



A



B



C

Plate 9 : Variation for inflorescence shape and size in *Trifolium*.

A. From L to R:

T. echinatum [EC 401714]

T. echinatum [EC 425077]

T. echinatum [EC 425076]

T. echinatum [EC 425078]

B. From L to R:

T. hybridum [EC 425032]

T. hybridum [EC 425030]

T. repens [EC 400986]

T. echinatum [EC 425076]

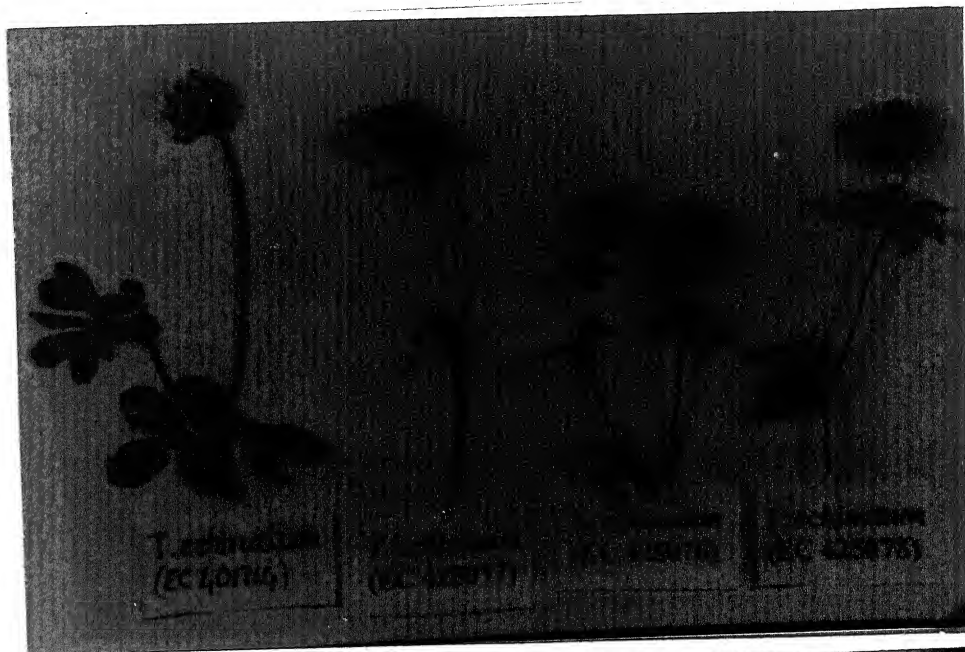
T. campestre [EC 425027]

C. From L to R:

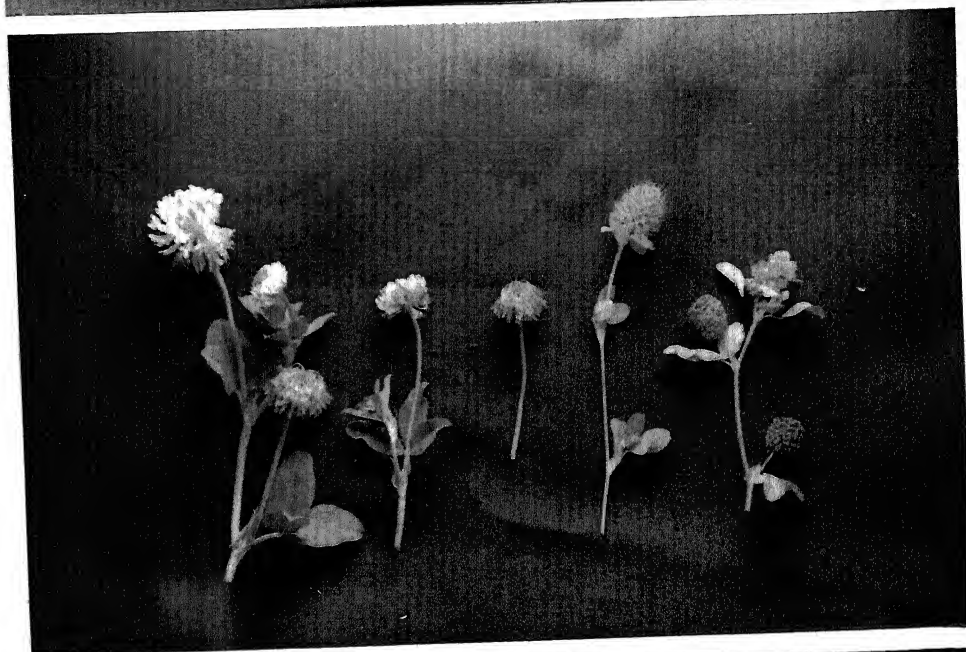
T. hirtum [EC 425038, EC 425037]

Plate : 9

trifolium.



A



B



C

Petioles of *T. incarnatum*, *T. resupinatum*, *T. arvense*, *T. constantinopolitanum*, *T. apertum*, *T. subterraneum* were observed to be quite long and ranged from 7 to 8.4 cm whereas petioles of *T. echinatum*, *T. campestre*, *T. angustifolium*, *T. tembense* were quite short ranging from 1.0 to 2.0 cm. *T. alexandrinum*, *T. hirtum*, *T. argutum*, *T. pratense*, *T. diffusum*, *T. glomeratum* and *T. hybridum* possessed medium sized petioles ranging from 5.0 to 6.4 cm.

Leaflets of *T. alexandrinum* were longest (3.7 cm) followed by that of *T. purpureum* (3.1 cm). *T. lappaceum*, *T. cherleri*, *T. hirtum*, *T. diffusum*, *T. spumosum*, *T. argutum*, *T. arvense*, *T. campestre*, *T. tembense*, *T. apertum*, *T. constantinopolitanum*, *T. repens*, *T. subterraneum*, *T. glomeratum*, *T. incarnatum*, *T. alpestre* and *T. echinatum* possessed small leaflets ranging from 0.8 to 1.8 cm. Leaflets of *T. incarnatum* were widest (1.6 cm) followed by that in three species viz. *T. alexandrinum*, *T. pratense*, *T. resupinatum* (1.5 cm) whereas minimum leaf breadth was noticed in *T. angustifolium* (0.4 cm). The species viz. *T. lappaceum*, *T. campestre*, *T. cherleri*, *T. argutum*, *T. echinatum*, *T. alpestre*, *T. purpureum*, *T. constantinopolitanum*, *T. hirtum*, *T. repens*, *T. glomeratum*, *T. medium*, *T. apertum*, *T. arvense*, *T. diffusum*, *T. spumosum* and *T. hybridum* possessed medium broad leaves (0.7 to 1.3 cm).

The fused portion of stipules in *T. purpureum* was longest (2.1 cm) followed by that in *T. alexandrinum* (1.8 cm). The fused portion of stipules in *T. campestre*, *T. argutum*, *T. incarnatum*, *T. tembense*, *T. repens*, *T. glomeratum*, *T. cherleri*, and *T. medium* was quite short ranging from 0.4 to 0.6 cm. Free portion of stipules in *T. purpureum*, *T. hybridum*, *T. alexandrinum* and *T. resupinatum* was observed to be quite long and ranged from 1.1 to 1.5 cm whereas the stipule of *T. lappaceum*, *T. campestre*, *T. incarnatum*, *T. cherleri* and *T. tembense* were quite short (± 0.4 cm). The longest total stipule length was observed in *T. purpureum* (3.6 cm) followed by 2.9 cm long stipules in *T. alexandrinum*. *T. campestre*, *T. tembense*, *T. argutum*, *T. glomeratum*, *T. incarnatum*, *T. cherleri*, *T. repens* and *T. medium* possessed small stipules ranging from 0.8 to 1.1 cm.

Morphological observation on floral parts of different species revealed that bracts were present in *T. glomeratum*, *T. repens*, *T. resupinatum*, *T. vesiculosum*, *T. spumosum*, *T. hybridum*, *T. nigrescens* and *T. retusum* whereas the other species of

study were ebracteate in nature. Calyx venation also revealed presence of 5 to 6 veins in *T. hybridum*, *T. repens* and *T. campestre* and more than 5 veins in rest of species.

4.1.3 Clustering of *T. alexandrinum* lines based on morphology

Clustering of fifty *T. alexandrinum* lines based on 16 characters was done using Euclidian cluster analysis method. A total of 8 clusters was observed (Table 4.7). Cluster number 1 and 6 were observed with maximum number of eight accessions followed by 7 accessions each in cluster number 3 and 7. Cluster number 2 and 8 were with least number of accessions i.e. 4 accessions each. Maximum distance was observed between cluster number 8 and 5 (6.57) followed by 6.26 inter cluster distance between cluster number 3 and 2 (Table 4.8). Cluster number 1 showed least distance with cluster number 6 i.e. 2.526 followed by 3.190 distance noticed between cluster number 6 and 7. Both the clusters 1 and 6 were found closer and with same number of accessions i.e. 8 accessions. Cluster number 1 was represented by two JHB accessions, one each from Rajasthan, Haryana and Punjab and three Warden lines whereas in cluster number 6 was represented by five JHB accessions, two Rajasthan accessions and one exotic accession (IL 40014).

4.1.4 Clustering of different accessions of *Trifolium* species based on morphology

Clustering of seventy five accessions belonging to 25 *Trifolium* species based on 8 morphological variants was done using Euclidian cluster analysis method. A total of nine clusters were observed (Table 4.9). Maximum 22 accessions were present in cluster number 5 followed with the seventeen accessions in cluster number 1. Cluster number 4 and 8 were represented by only 2 accessions each. The maximum distance was observed between cluster number 8 and 9 (i.e. 7.46) followed by intercluster distance of cluster 8 with clusters 1 and 2 (Table 4.10). Cluster number 9 and 1 showed least distance of 1.74 followed by 1.99 between cluster 5 and 1. All nine *T. alexandrinum* accessions formed a separate cluster whereas in cluster number 4, two accessions of *T. purpureum* EC 425069 and EC 425070 were present.

Table 4.7. Clustering of *T. alexandrinum* genotypes based on morphology

Cluster Number	Genotypes
1	JHB 15-27, JHB -P- 23/35, Raj 7/13-25, HFB 155, BL 131, Wardan S-1, Wardan S-2, Wardan S-3
2	IL 40010-Mes, JHB 91P-20, JHB 34/22, JHTB-1-90-A1
3	JHB 94-R-16, JHB-R-35, JHB 6/54 p/t, JHB 146, Raj 7/49-50, IL 4009, Wardan S-4
4	JHB 94-R-13, JHB 94-R-25, JHB 94-P-60, BL 144, JHB 94-56
5	JHB 57 P3, JHB 5-13/12, Raj 7/53-54, Raj 7/53-54-O, Raj 7/53-54-2, Raj 7/13-14-O, JHB 36/5-54
6	JHB 94P/T 34, JHB P 17-1, Raj 7/13-14, JHB 94-18/11, Raj Bundi - O, JHB 94-25, IL 40014, JHB 94-5
7	Wardan, JHB 94 P-22, JB 92-1, BL 122, JHTB 9-90 N1, JHB16/2, HFB 155
8	JHB 94-31, IL40013, JHB CT2-6/35, JHB 6/54

Table 4.8. Intercluster distances among different genotypes of *T. alexandrinum*

Cluster No.	1	2	3	4	5	6	7	8
1	0.000							
2	3.211	0.000						
3	4.417	6.262	0.000					
4	3.954	4.071	4.036	0.000				
5	3.566	5.356	3.386	5.076	0.000			
6	2.526	3.537	4.049	3.802	3.519	0.000		
7	3.832	5.038	3.808	3.635	4.155	3.190	0.000	
8	4.439	4.729	6.155	4.749	6.567	3.511	4.699	0.000

Table 4.9 Clustering of different accessions of *Trifolium* species based on morphology

Cluster	Species	Accession no.
1	<i>T. tembense</i>	EC 402169
	<i>T. lappaceum</i>	EC 402165
	<i>T. argutum</i>	EC 402154
	<i>T. campestre</i>	EC 402155
	<i>T. repens</i>	EC 401708, EC 400985, EC 400986
	<i>T. hybridum</i>	EC 401702, EC 401701
	<i>T. glomeratum</i>	EC 402170
	<i>T. cherleri</i>	EC 401703
	<i>T. echinatum</i>	EC 425075
	<i>T. alpestre</i>	EC 425043, EC 425042
	<i>T. hirtum</i>	EC 425037
	<i>T. angustifolium</i>	EC 425062, EC 425061
2	<i>T. alexandrinum</i>	Wardan, EC 401711, EC 400976, EC 400977, EC 402161, JHB 146, EC 401709, EC 401710, EC 400733
3	<i>T. echinatum</i>	EC 425076, EC 425077, EC 425078
	<i>T. campestre</i>	EC 425028, EC 425026
4	<i>T. purpureum</i>	EC 425069, EC 425070
5	<i>T. diffusum</i>	EC 402163
	<i>T. spumosum</i>	EC 402160
	<i>T. arvense</i>	EC 402156
	<i>T. pratense</i>	EC 401719, EC 400735, EC 400982, PRC-3, EC 400980, EC 400979, EC 402168
	<i>T. subterraneum</i>	EC 401718, EC 401717, EC 402167, IG 96-112, IG 96-113,
	<i>T. glomeratum</i>	EC 401700, EC 402170, EC 425033
	<i>T. incarnatum</i>	EC 402164, IG 96-111
	<i>T. hybridum</i>	EC 425030, EC 425032
	<i>T. hybridum</i>	EC 425029,
	<i>T. nigrescens</i>	EC 425047
6	<i>T. resupinatum</i>	SH 98-73, SH 98-86, SH 98-15
	<i>T. hirtum</i>	EC 402153, EC 425039
7	<i>T. apertum</i>	EC 401712
	<i>T. constantinopolitanum</i>	EC 401713
	<i>T. nigrescens</i>	EC 425049, EC 425048
	<i>T. resupinatum</i>	SH 98-36, SH 98-72
9	<i>T. pratense</i>	EC 401721, EC 401720
	<i>T. medium</i>	EC 425045
	<i>T. tembense</i>	EC 425064, EC 425066, EC 425065
	<i>T. campestre</i>	EC 425027

Table 4.10 Inter cluster distances among different accessions of *Trifolium*

Cluster No.	1	2	3	4	5	6	7	8	9
1	0.000								
2	4.113	0.000							
3	3.479	5.472	0.000						
4	4.598	2.634	5.157	0.000					
5	1.996	3.674	4.250	5.006	0.000				
6	4.557	2.830	5.240	4.092	3.181	0.000			
7	2.062	4.094	3.211	4.215	2.502	3.559	0.000		
8	6.199	6.199	4.121	5.464	6.144	4.845	4.546	0.000	
9	1.746	5.287	4.024	5.982	2.856	5.781	3.637	7.455	0.000

Out of 5 accessions of *T. resupinatum* three accessions (SH 98-73, SH 98-86 and SH 98-15) were present with single accession each of *T. hybridum* (EC 425029) and *T. nigrescens* (EC 425047) in cluster number 6. Remaining two accessions of *T. resupinatum* (SH 98-36 and SH 98-72) made a separate cluster number 8.

Five accessions of *T. hybridum* showed wide variation among themselves. Two accessions (EC 401702 and EC 401701) were present with 3 accessions of *T. repens* (EC 401708, EC 400985, EC 400986) and single accessions of *T. glomeratum* (EC 402170) in cluster number 1 whereas two accessions of *T. hybridum* (EC 425030 and EC 425032) were present in cluster number 5 with *T. incarnatum* (EC 402164, IG 96-111) and three accessions of *T. glomeratum* (EC 401700, EC 402170, EC 425033). *T. hybridum* (EC 425029) was present in cluster number 6 with single accession of *T. nigrescens* (EC 425047) and three accession of *T. resupinatum* (SH 98-73, SH 98-86 and SH 98-15). Nine accessions of *T. pratense* were present in two cluster i.e. in cluster number 5 and 9. In cluster number 9, only 2 accessions of *T. pratense* (EC 401721 and EC 401720) were present with *T. medium* (EC 425045), *T. tembense* (EC 425064, EC 425066, EC 425065) and *T. campestre* (EC 425027).

Cluster number 2 of 9 accessions of *T. alexandrinum* showed the maximum distance i.e. 6.199 with cluster number 8 in which only two accessions of *T. resupinatum* (SH 98-36 and SH 98-72) were present whereas it showed minimum distance 2.634 from cluster number 4 comprising of 2 accessions of *T. purpureum* (EC 425069 and EC 425070).

4.2 Cytological studies

Chromosome behaviour during meiosis was observed in Pollen Mother Cells (PMCs) at diakinesis. Types of chromosomal associations and meiotic abnormalities observed in different species are as follows (Table 4.11, Plate 10 (A to H)):

T. hybridum – The diploid chromosome number was found to be 16 in both the accessions EC 425030 and EC 425032. Normal meiotic behavior was observed. Most of the cells had 8 bivalents with occasional presence of single quadrivalents in few PMCs. On an average 7.9 and 7.6 bivalents per cells were recorded in EC

Plate : 10

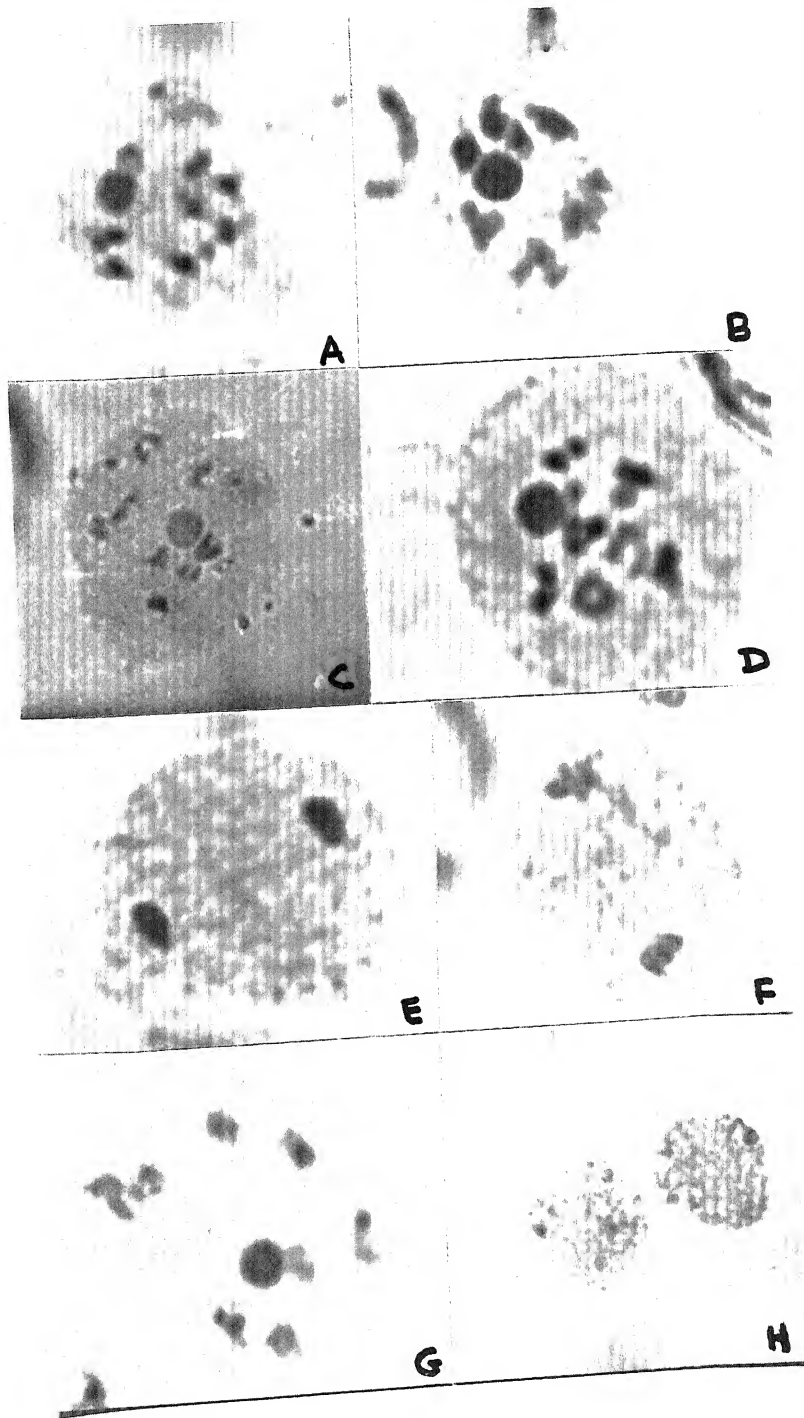


Plate 10 : Meiotic behavior in PMCs of different *Trifolium* species.

- A. 8 IIs, 2 nucleoli (one small and one large) in *T. hirtum* [EC 402153]
- B. 8 IIs, 2 rod + 6 ring in *T. hirtum* [EC 402153]
- C. 8 IIs, 4 rod + 4 ring in *T. resupinatum* [JHS-3]
- D. 6 IIs+ 1 IV in *T. resupinatum* [SH 99-69]
- E. Anaphase I in *T. resupinatum* [JHS-3]
- F. Anaphase I showing lagging chromosome in *T. resupinatum* [SH 99-69]
- G. 8 II in *T. hybridum* [EC 425032]
- H. Transmigration of genetic material in *T. campestre* [EC 402155]

Table 4.11. Chromosomal associations in different species of *Trifolium*

Species	PMCs observed	2n=	Chromosomal association				Other observations	Pollen fertility (%)
			I	II	III	IV		
<i>T. hybridum</i> EC 425030	25	16	-	6-8 (7.88)	-	0-1 (0.06)		95.4
<i>T. hybridum</i> EC 425032	25	16	0-1 (0.12)	6-8 (7.6)	0-1 (0.12)	0-1 (0.08)		98.2
<i>T. resupinatum</i> JHS-3	25	16	0-2 (0.15)	7-8 (7.92)	-	-	Two nucleoli, transmigration, multiple spindle	97.2
<i>T. vesiculosum</i> EC 401716	25	16	-	8 (8.0)	-	-	-	98.7
<i>T. incarnatum</i> EC 402164	25	14	0-4 (1.8)	5-7 (6.1)	-	-	Unequal distribution, lagging chromosomes	63.0
<i>T. hirtum</i> EC 402153	25	16	0-2 (0.8)	7-8 (7.6)	-	-	Two nucleoli, one big & one small, 2 II s attached to different nucleoli	86
<i>T. campestre</i> EC 402155	25	14	-	7 (7.0)	-	-	Transmigration, multiple spindle	85.2
<i>T. constantinopoli</i> <i>tanum</i> EC 401713	25	16	0-2 (0.2)	7-8 (7.9)	-	-		100
<i>T. alexandrinum</i> Wardan	25	16	-	8(8.0)	-	-		95.0
<i>T. cherleri</i> EC 401703	25	10	-	5(5.0)	-	-		98.8
<i>T. purpureum</i> EC 425069	25	14	0-2 (0.3)	6-7 (6.85)	-	-		97.8

425030 and EC 425032 respectively. Occasional presence of trivalents was noticed in EC 425032.

T. resupinatum (JHS-3)- Meiosis was found to be normal in most of the 25 PMCs studied. Regular pairing of homologous chromosomes resulted in 7-8 bivalents per PMC. An average of 7.9 bivalents per cell was noticed. A few cells were observed with two univalents. Presence of two nucleoli attached to different bivalents was noticed at diakinesis stage. Transmigration of genetic material from one PMCs to other was quite common. Multiple spindle formation was also observed in this species. Pollen stainability ranged from 95 to 100%.

T. vesiculosum (EC 401716): Normal meiosis with eight bivalents per cell was observed in this species. Pollen stainability was 98%.

T. incarnatum (EC 402164): Meiosis was found to be more or less normal in most of the PMCs and formation of 5 to 7 bivalents was observed. On an average, 6.1 bivalents per cell was noticed. A few cells were observed for presence of 4 univalents. Unequal distribution of chromosome was also noticed. Lagging chromosomes were also seen in anaphase I in few PMCs. Pollen stainability was 63%.

T. hirtum (EC 402153): Normal meiosis was noticed in majority of the PMCs and 7-8 bivalents per cell were observed. Average bivalent per cell was 7.6. A few cell were observed with two univalents. Presence of one big and one small nucleolus in the PMCs was also noticed and one bivalent was attached to each nucleoli. The pollen fertility was -86 %.

T. campestre (EC 402155): Diploid chromosome number was found to be $2n=14$. Meiosis in this species was normal. Regular pairing of homologous resulted in 7 bivalents. Transmigration of genetic material from one to other PMC was observed. Multiple spindle formation at metaphase was also noticed. Pollen fertility ranged from 80-90% among different plants.

T. constantinopolitanum (EC 401713): Meiosis in this species was normal. Pairing of homologous chromosomes resulted in 7-8 bivalents with an average of 7.9 bivalents per cell. A few PMCs were noticed with 2 univalents. Pollen fertility was found to be normal.

T. alexandrinum (Wardan): Normal meiosis was observed in this species. Pairing of homologous chromosomes resulted in 8 bivalents. Pollen fertility was 95 %.

T. cherleri (EC 401703): The diploid chromosome number was found to be $2n=10$. Normal meiosis was noticed and pairing of homologous chromosomes resulted in 5 bivalents per cell. Pollen fertility was recorded to be 98.8%.

T. purpureum (EC 425069): Normal meiosis was observed in most of the PMCs. Pairing of homologous chromosomes resulted in 6-7 bivalents per cell. A few cells were observed with 2 univalents.

4. 3. Isozyme studies

The present study was undertaken to estimate the genetic diversity among species of the genus *Trifolium* for five enzyme systems, i.e., Esterase, SOD, GOT, ACP and Peroxidase. One hundred thirty four accessions belonging to 25 different species of genus *Trifolium* were subjected to horizontal starch gel electrophoresis using discontinuous buffer system. The unambiguous bands were scored and numbered on the basis of their relative mobility towards anodal / cathodal ends (Table 4.12).

4.3.1 Banding Pattern of different enzymes

Esterase : The study showed presence of six distinct migration zones with a total of 18 bands distributed through these six zones. Slowest zone was with single band whereas fastest zone was most polymorphic represented by five bands. Two bands each in second and fifth migration zone and four each in third and fourth migration zones were identified. Wide variation in relative mobility (RM) of band was observed which ranged from 0.12 to 0.96 RM (Table 4.12; Fig 10).

Superoxide Dismutase (SOD): The study showed the presence of eight bands distributed through three distinct migration zones. There were two bands each in first and second zone whereas four bands were identified in the third zone. The relative mobility (RM) varied from 0.49 to 0.9 RM (Table 4.12; Fig 11).

Glutamate Oxaloacetic Transaminase (GOT) : GOT isozyme banding pattern in *Trifolium* revealed the presence of three migration zones and a total of ten bands distributed through these three zones. First zone was represented by three bands,

Table 4.12. RM values for bands of various enzymes observed in *Trifolium*

Enzyme	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Esterase	0.12	0.23	0.28	0.33	0.39	0.46	0.50	0.55	0.58	0.61	0.63	0.72	0.77	0.83	0.87	0.90	0.93	0.96
SOD	0.49	0.52	0.70	0.73	0.81	0.85	0.87	0.9										
GOT	0.11	0.17	0.21	0.31	0.33	0.39	0.44	0.47	0.61	0.65								
ACP	0.45	0.58	0.63	0.69	0.73	0.78	0.84	0.88										
Perox (A)	0.15	0.23	0.27	0.32	0.38	0.42	0.48	0.54	0.59	0.71	0.92							
Perox (C)	0.15	0.20	0.33	0.42	0.49	0.58	0.63	0.74	0.79	0.84	0.92							

SOD=Super Oxide Dismutase GOT= Glutamate Oxaloacetate Transaminase

ACP= Acid phosphatase Perox (A)= Peroxidase anodal

Perox (C)= Peroxidase cathodal

Fig 10. Zymogram for Esterase in different *Trifolium* species

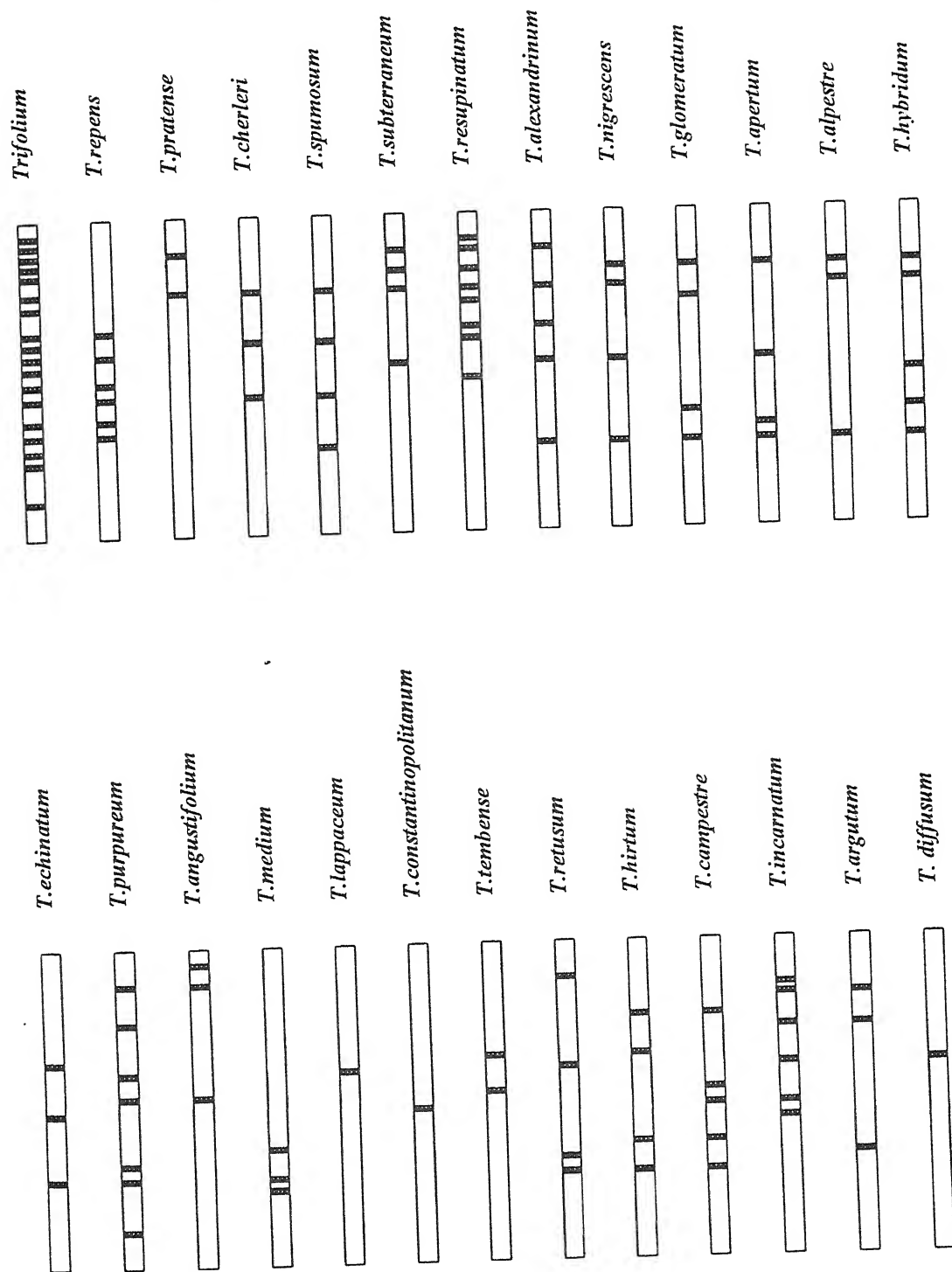
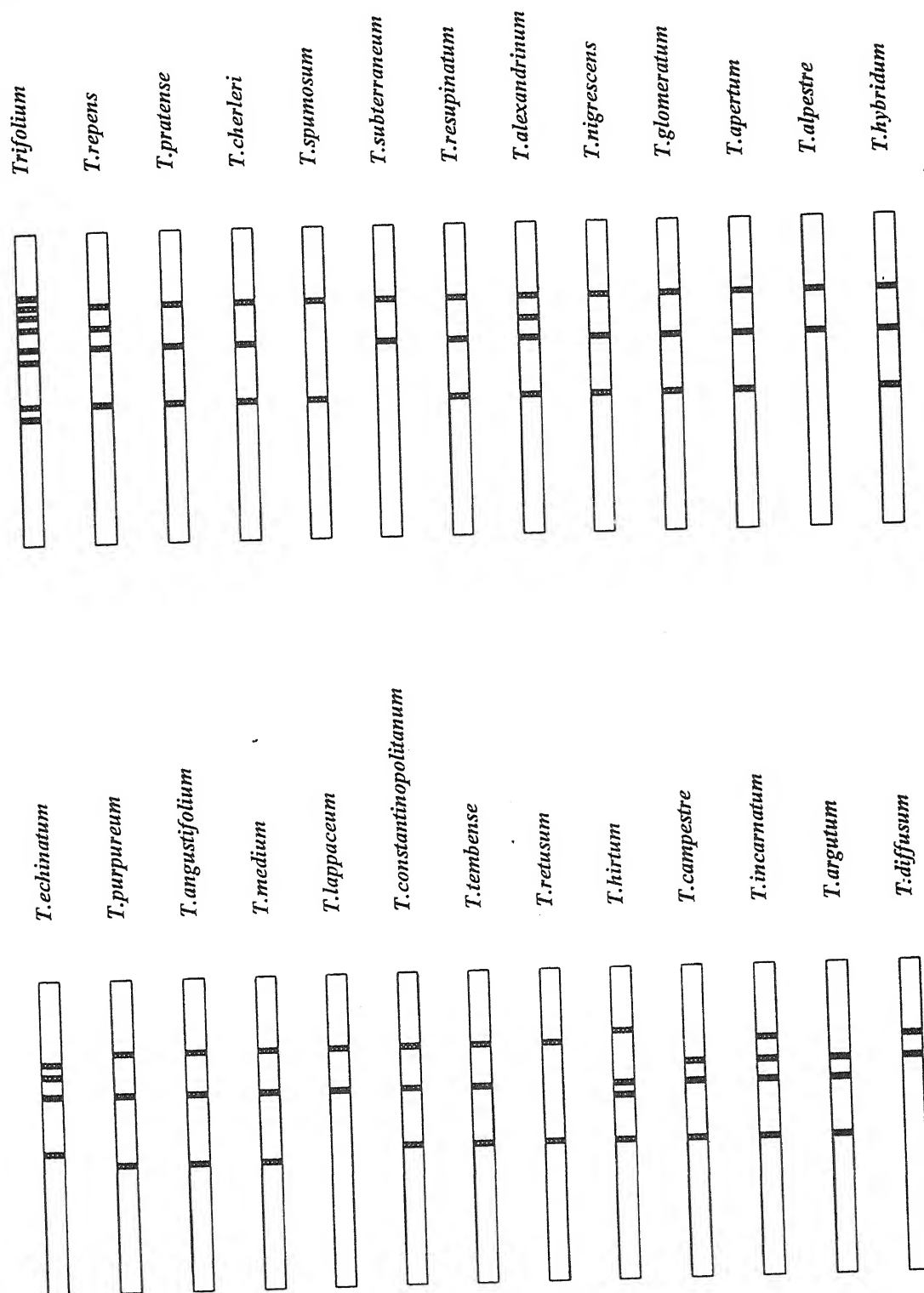


Fig. 11. Zymogram Pattern for Super Oxide Dismutase in different *Trifolium* species



second by five bands and the third by two bands. The relative mobility (RM) of bands ranged from 0.11 to 0.65 (Table 4.12; Fig 12).

Acid Phosphatase (ACP): The study revealed the presence of eight bands in three distinct migration zones. In the slowest zone one band was present whereas five and two bands were noticed in second and third migration zones respectively. The slowest band was observed at 0.45 RM and the fastest at 0.88 RM (Table 4.12; Fig 13).

Peroxidase: The study revealed the presence of eight peroxidase migration zones (five towards anodal side and three towards cathodal side). Anodal bands were prefixed with A and Cathodal bands with C for identification. Eleven bands were present on either sides. Towards anodal side, Zone 1, 4 and 5 were represented by single band whereas 3 and 5 bands were present in zone two and zone five respectively. At cathodal end three zones were identified which were represented by 2, 5 and 4 bands respectively (Table 4.12; Fig 14).

4.3.2 Intra species isozyme variations:

4.3.2.1. Intraspecies diversity for Esterase:

In all the nine accessions of *T. repens* Band 11 was invariably present. Band 4 was present only in five accessions. Band 5 was represented only in EC 401708. Band 6, 7 and 9 were present in two accessions namely EC 401707 and EC 401704. Five accessions with only two bands were found to be similar (Table 4.13). In EC 401705 single band no. 11 was observed.

Eight accessions of *T. pratense* were studied. Out of total 18 bands, only two bands i.e. Band 13 and 16 were represented. Of these two bands, Band 13 was invariably present in all the accessions, while Band 16 was absent in EC 400979, PRC-3, EC 400980. In all five accessions of *T. subterraneum* three bands viz. Band 8, 13 and 14 were invariably present in addition to band 16 present only in EC 401718. In all three accessions of *T. nigrescens*. Band 3 and 13 were present in addition to Band 8 in EC 425048 and Band 14 in EC 425047. Band 3, 5 and 11 were common to all three accession of *T. hirtum*, whereas an additional band No 13 was present in only two accessions viz. EC 425038 and EC 425037.

Fig.12. Zymogram pattern for Glutamate Oxaloacetate Transaminase in different *Trifolium* species

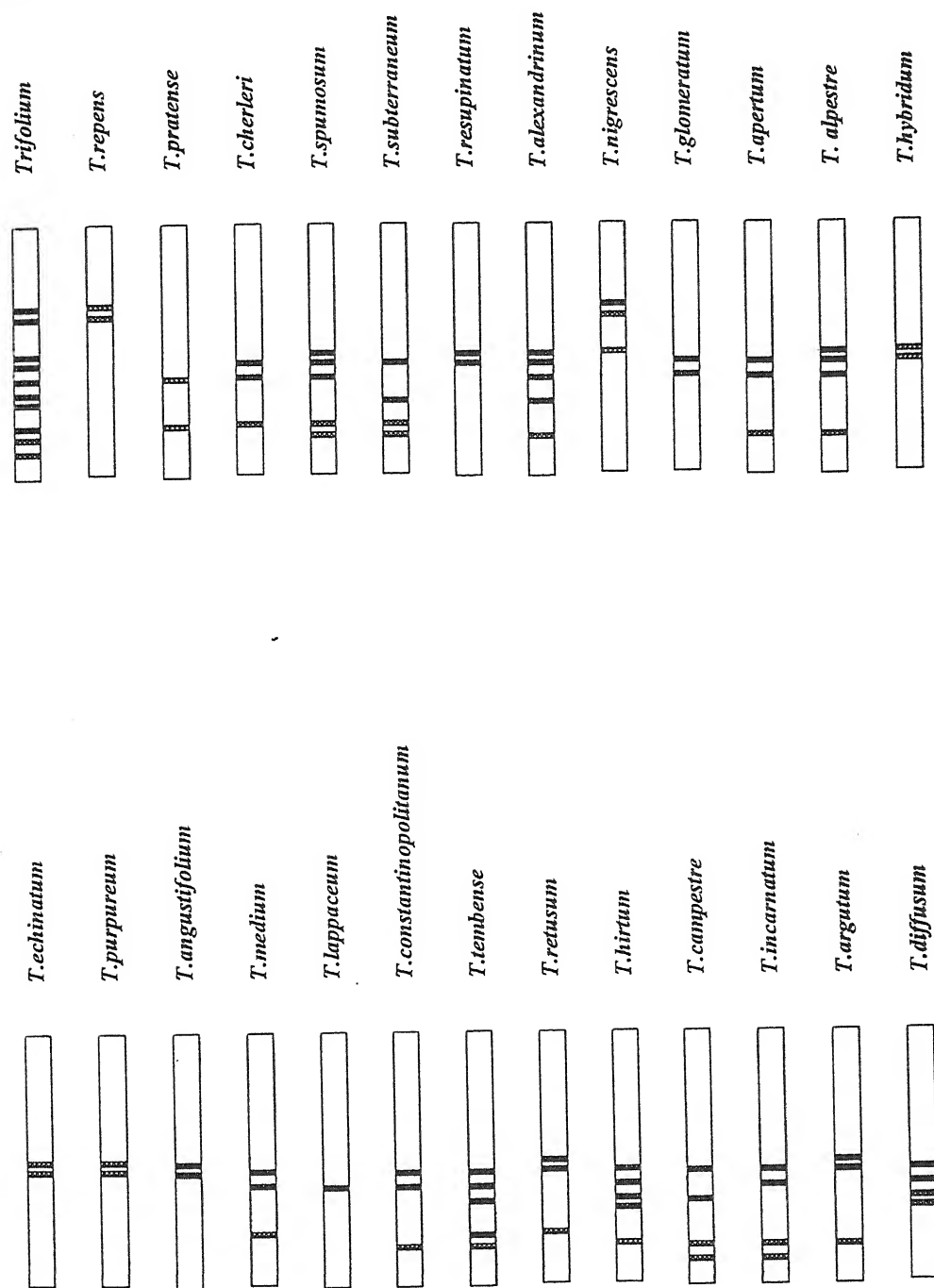


Fig.13. Zymogram for Acid Phosphatase in different *Trifolium* species

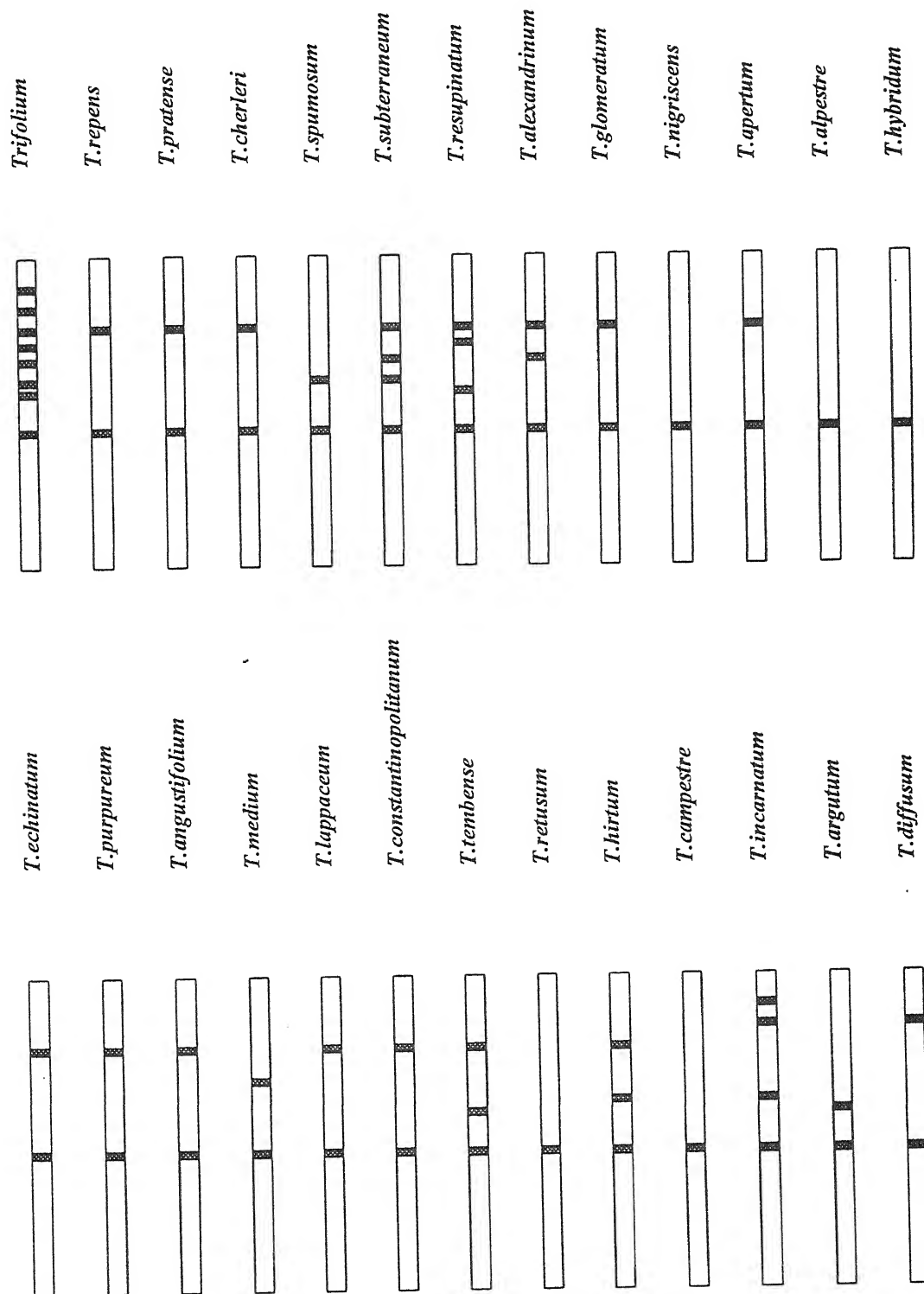


Fig. 14. Zymogram pattern for peroxidase in different *Trifolium* species

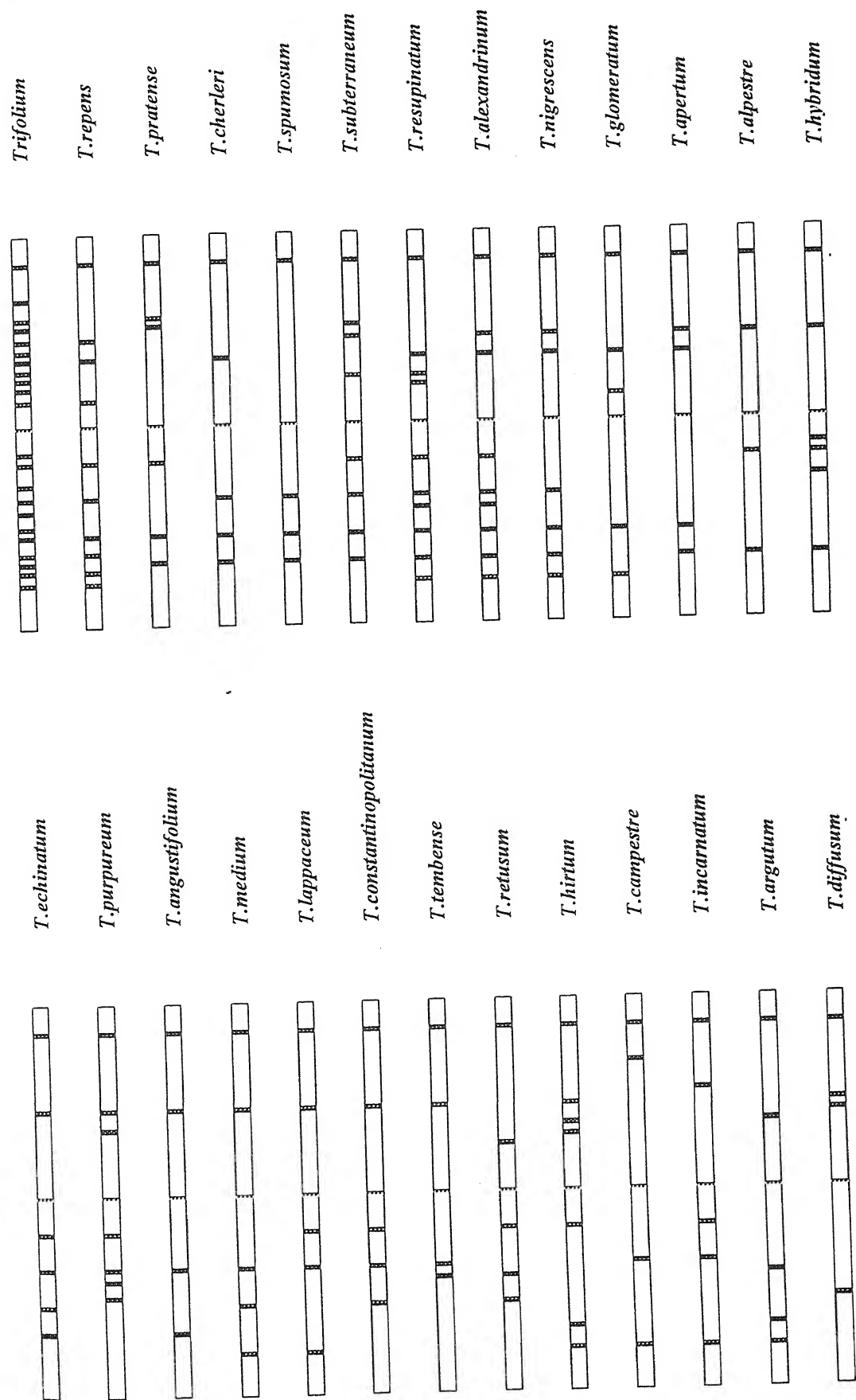


Table 4.13. Zymogram pattern for Esterase among different *Trifolium* species

Species	Bands	Accession No.	Accessions
<i>T. repens</i>	6, 7, 9, 11	2	EC 401707, EC 401704
	5, 11	1	EC 401708
	11	1	EC 401705
	4, 11	5	EC 401706, EC 400985, EC 400986, EC 400984, EC 400987
<i>T. pratense</i>	13	3	EC 400979, PRC-3, EC 400980
	13, 16	5	EC 400982, EC 401721, EC 401719, EC 401720, EC 400735
<i>T. retusum</i>	3, 4, 10, 16	1	EC 402150
<i>T. argutum</i>	4, 12, 14	1	EC 402154
<i>T. spumosum</i>	3, 6, 10, 13	1	EC 402160
<i>T. tembense</i>	8, 11	1	EC 402169
<i>T. apertum</i>	3, 4, 8, 14	1	EC 401712
<i>T. lappaceum</i>	10	1	EC 402165
<i>T. subterraneum</i>	8, 13, 14	4	IG 96-112, IG 96-113, EC 402167, EC 401717
	8, 13, 14, 16	1	EC 401718
<i>T. nigrescens</i>	3, 8, 13	1	EC 425048
	3, 13, 14	1	EC 425047
	3, 13	1	EC 425049
<i>T. hirtum</i>	3, 5, 11, 13	2	EC 425038, EC 425037
	3, 5, 11	1	EC 425039
<i>T. hybridum</i>	5, 7, 13	2	EC 401702, EC 401701
	5, 13	1	EC 425032
	3, 7, 14	1	EC 425029
	7	1	EC 425030
<i>T. diffusum</i>	10	1	EC 402163
<i>T. alpestre</i>	3	1	EC 425042
	3, 13, 14	1	EC 425043
<i>T. echinatum</i>	3, 7, 11	2	EC 401714, EC 425076
<i>T. purpureum</i>	1, 3, 4, 8, 10, 13	1	EC 425069
	1, 4, 8, 10, 13, 16	1	EC 425070
<i>T. campestre</i>	3, 5, 8, 13	1	EC 425028
	3, 5, 7, 13	1	EC 425026
<i>T. incarnatum</i>	6, 7, 12, 14, 15	1	IG 96-111
	6, 7, 10, 12, 14, 15	1	EC 402164
<i>T. cherleri</i>	6, 10, 13	1	EC 401703
<i>T. resupinatum</i>	13, 16	1	SH 98-36
	12, 13	1	SH 98-72
	10, 16, 17	1	SH 98-73
	10, 16	1	SH 98-86
	10, 12, 13, 16, 17	1	JHS-3
	7, 10, 14	1	SH-99-29
	10, 11, 14	3	SH-99-65, SH-99-23, SH-99-33
	11, 13, 14	1	SH-99-32
	7, 12, 14	1	SH-99-25
	11, 12, 14	1	SH-99-26
<i>T. angustifolium</i>	8, 16, 18	2	EC 425062, EC 425061
<i>T. medium</i>	2, 3, 5	1	EC 425045
<i>T. constantinopolitanum</i>	7	1	EC 401713
<i>T. glomeratum</i>	3, 5, 12, 14	2	EC 402170, EC 401700

Band 3 was found in both the accessions of *T. alpestre*. EC 425043 was marked for two additional bands (Band 13 and 14). Band 3, 7 and 11 were present in both accessions of *T. echinatum*. Band 1, 4, 8, 10 and 13 were common in both accessions of *T. purpureum* with an additional Band 3 in EC 425069 and band 16 in EC 425070. In *T. campestre* Band 3, 5 and 13 were present in both accessions whereas Band 7 and 8 were present in EC 425026 and EC 425028 respectively.

Five bands, i.e. Band 6,7,12,14 and 15 were present in both the accessions of *T. incarnatum* in addition to Band 10 present only in EC 402164. In *T. cherleri*, Band 6, 10 and 13 were present. *T. lappaceum* and *T. diffusum* were marked for presence of single band i.e. Band 10. Marked diversity for esterase isozyme pattern was observed in twelve accessions of *T. resupinatum*. Band 7 was present in only two accessions i.e. SH-99-29 and SH-99-25. Band 10 was represented in 7 accessions. Band 17 was present only in two accessions i.e. SH 98-73 and JHS-3. The maximum number of 5 bands were present in JHS-3 whereas minimum only two bands were present in SH 98-36, SH 98-72 and SH 98-86. Only three bands i.e. Band 8, 16 and 18 were present in both accessions of *T. angustifolium*. In single accession of *T. medium* Band 2, 3 and 5 were present.

In *T. constantinopolitanum* only Band 7 was noticed in single accession studied. Two accessions of *T. glomeratum* i.e. EC 402170 and EC 401700 were studied and the four bands viz. Band 3, 5, 12 and 14 were common to both the accessions. In *T. retusum*, Bands 3, 4, 10 and 16 were present. Bands 4, 12 and 14 were present in single accession of *T. argutum*. In *T. spumosum* Bands 3, 6, 10 and 13 were present. Bands 8 and 11 were present in *T. tembense*. In *T. apertum*, four bands i.e. Band 3, 4, 8 and 14 were present.

Five accessions of *T. hybridum* were studied. The number of bands in different accessions ranged from 1 to 3. In EC 425030 only Band 7 was present. Band 3 and 14 were present only in EC 425029. Band 5 was present in three accessions viz. EC 401702, EC 401701 and EC 425032.

Analysis of 65 accessions of *T. alexandrinum* representing exotic lines, Fahli and 'Saidi' types, pentafoliate lines, red flowered, open and self pollinated plants, lines from Rajasthan, Punjab and Hisar showed no genetic diversity for esterase

**Plate 11 : Variation for esterase isozyme banding pattern in
different accessions of *Trifolium* species.**

A. From L to R.

T. alexandrinum

Raj 7/13-14 -O, JHTB 5-90-I, IL 40010, JHB 94 -18/11, JHB 91 P-20,
JHB 34/22, Raj-Bundi-O, JHB -P-23/35, JHTB-1-90-A1, JHB 94-31,
JHB 94-25

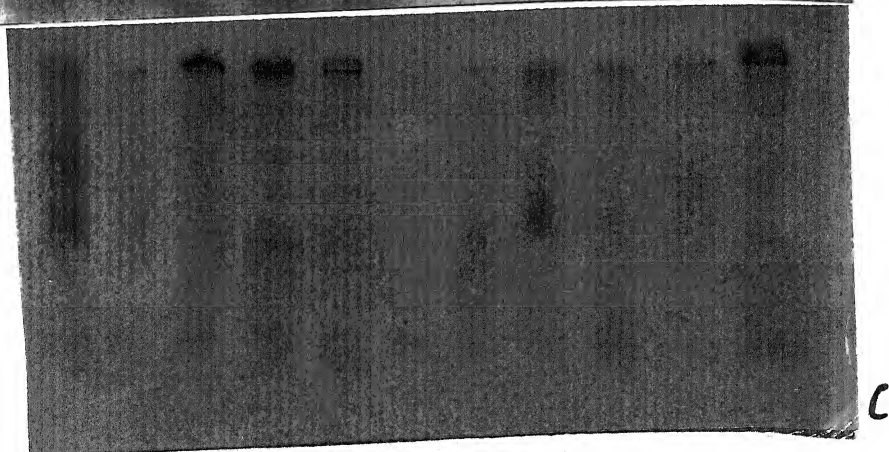
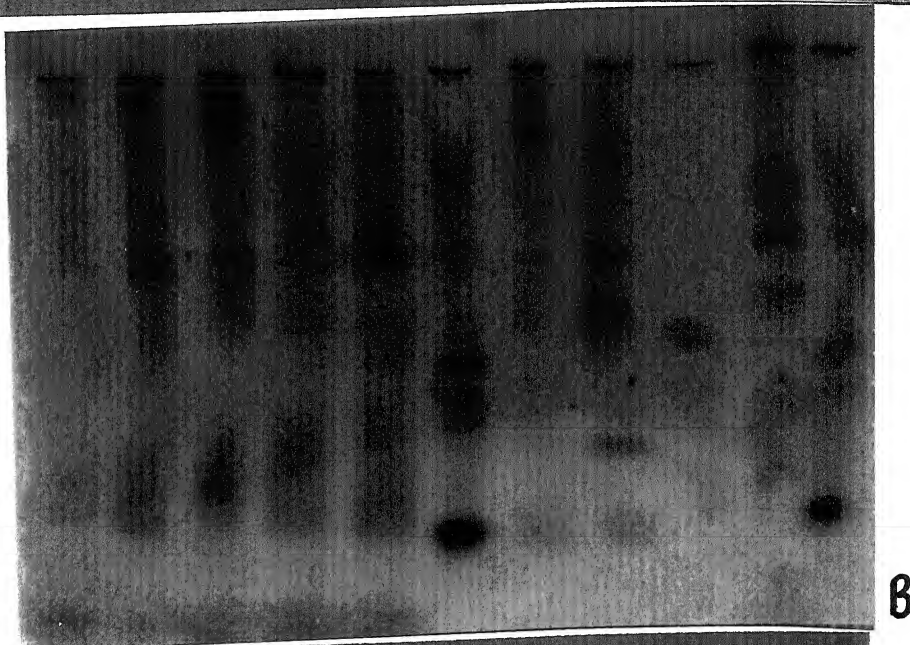
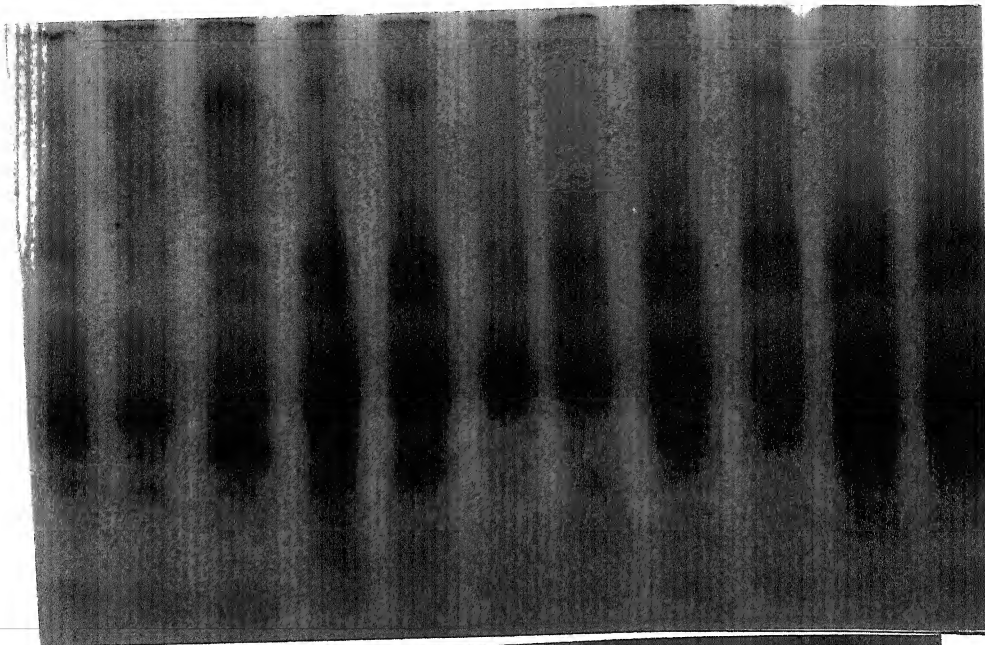
B. From L to R.

<i>T. subterraneum</i> [EC 401718],	<i>T. subterraneum</i> [EC 401717],
<i>T. subterraneum</i> [EC 402167],	<i>T. subterraneum</i> [IG 96-112],
<i>T. subterraneum</i> [IG 96-113],	Test sample
<i>T. incarnatum</i> [EC 402164],	<i>T. incarnatum</i> [IG 96-111],
Test sample	<i>T. cherleri</i> [EC 401703] ,
Test sample	

C. From L to R.

<i>T. cherleri</i> [EC 401703],	<i>T. repens</i> [EC 400987],
<i>T. alexandrinum</i> [JHTB 1-90A1],	<i>T. alexandrinum</i> [Wardan],
<i>T. alexandrinum</i> [Wardan],	<i>T. repens</i> [EC 401704],
<i>T. echinatum</i> [EC 401714],	<i>T. repens</i> [EC 400984],
<i>T. repens</i> [EC 400987],	<i>T. repens</i> [EC 400984],
<i>T. alexandrinum</i> [JHTB 1-90A1],	

Plate : 11



isozymes. Five bands (Band 3, 8, 11, 13 & 16) were present in all the accessions (Table 4.14).

4.3.2.2. Intraspecies variation for Superoxide Dismutase (SOD)

Nine accessions of *T. repens* were studied for SOD enzyme system. Bands 2 and 4 were common in all nine accessions. Band 7 was represented in 7 accessions while absent in accession EC 401708 and EC 401705. In these two accessions Band 5 was present (Table 4.15). Out of total 8 bands, only three bands (Band 2, 4 and 7) were invariably present in all eight accessions of *T. pratense*. In *T. subterraneum* five accessions were analyzed and two bands i.e. Band 4 and 7 were present in all the accessions. Thus, no variation was found among different accessions of *T. subterraneum* and *T. pratense*.

Three accessions viz. EC 425048, EC 425047 and EC 425049 of *T. nigrescens* were analysed. Only two bands were present in each accession. Band 7 was common to all the three accession. Band 2 was present only in EC 425047 while band 4 was found in EC 425048 and EC 425049. In *T. hirtum* Band 2 and 8 were common to all the three accession studied, whereas Band 3 was present in EC 425038 and EC 425037 and absent in EC 425039. Band 4 was present only in EC 425039. Accessions EC 425037 and EC 425038 were found to be similar for all three bands. Single accession of *T. cherleri* (EC 401703) possessed three bands i.e. Band 2, 4 and 7. In *T. lappaceum* (EC 402165) only two bands (Band 4 and 7) were noticed. *T. diffusum* (EC 402163) also showed presence of only two bands i.e. Band 5 and 7.

In *T. alpestre*, Band 4 and 7 were found common in both the accessions. The two accessions of *T. purpureum* showed presence of Bands 1, 4 and 7. In two accessions of *T. campestre* Band 2 and 5 were present with an additional Band 4 in EC 425026. Two accessions of *T. incarnatum* (IG 96-111 and EC 422164) showed presence of four bands (Band 2, 4, 5 and 7).

All the 12 accessions of *T. resupinatum*, one each of *T. constantinopolitanum* and *T. apertum*, five of *T. hybridum* and two of *T. glomeratum* showed similar banding pattern with presence of Band 2, 4 and 7. Band 1,4 and 7 were found to be common in both EC 425062 and EC 425061 of *T. angustifolium* and *T. medium*.

Table 4.14. Zymogram pattern for Esterase among different genotypes of *T. alexandrinum*

Enzyme	Bands	No. Genotypes	Genotypes
Esterase	3, 8, 11, 13, 16	65	EC 329299, IL 40010, EC 400733, EC 401710, EC 401709, Wardan, EC 402161, EC 400977, EC 400976, EC 401711, JHB 94P-22, JHB94-R-16, JHB94-R-35, JHB94-R-13, JHB94-R-25, JHB94P/T-34, EC 318951, JHB 57P3, JHB P17-1, Raj 7/13-14, JHB 15-27, JHB 6/54 p/t, JB92-1, BL 122, JHB 146, Raj 7/49-50, JHTB 9-90 N1, JHB 5-13/12, IL 4009, JHTB 5-90-2, JHTB 3-90-H, JHTB 13-90-B, Raj 7/ 53-54, JHTB-1-90-P3, Raj 7/53-54-O, Raj 7/53-54-2, Raj 7/13-14-O, JHTB 5-90-I, IL 40010-Mes, JHB 94-18/11, JHB 91 P20, JHB 34/22, Raj-Bundi-O, JHB -P-23/35, JHTB -1-90-A1, JHB 94-31, JHB94-25, IL 40014, IL 40013, JHB 94-P-60, BL 144, JHB 94-56, BL 142, Raj 7/13-25, HFB 155, BL 131, JHB 36/5-54, JHB CT2 6/35, JHB 6/54, JHB 16/2, HFB 155, Wardan S-1, Wardan S-2, Wardan S-3, Wardan S-4,

Table 4.15. Zymogram pattern for SOD among different accessions of *Trifolium* species

Species	Bands	No. Accessions	Accessions
<i>T. repens</i>	2, 4, 7	7	EC 401707, EC 401704, EC 401706, EC 400985, EC 400986, EC 400984, EC 400987
	2, 4, 5	2	EC 401708, EC 401705
<i>T. pratense</i>	2, 4, 7	8	EC 400979, PRC-3, EC 400980, EC 400982, EC 401721, EC 401719, EC 401720, EC 400735
<i>T. subterraneum</i>	4, 7	5	IG 96-112, IG 96-113, EC 402167, EC 401717, EC 401718
<i>T. nigrescens</i>	4, 7	2	EC 425048, EC 425049
	2, 7	1	EC 425047
<i>T. hirtum</i>	2, 3, 8	2	EC 425038, EC 425037
	2, 4, 8	1	EC 425039
<i>T. diffusum</i>	5, 7	1	EC 402163
<i>T. hybridum</i>	2, 4, 7	5	EC 425032, EC 425029, EC 425030, EC 401702, EC 401701
<i>T. alpestre</i>	4, 7	2	EC 425042, EC 425043
<i>T. tembense</i>	2, 4, 7	1	EC 402169
<i>T. echinatum</i>	2, 4	1	EC 401714
	5, 6	1	EC 425076
<i>T. spumosum</i>	2, 7	1	EC 402160
<i>T. lappaceum</i>	4, 7	1	EC 402165
<i>T. purpureum</i>	1, 4, 7	2	EC 425069, EC 425070
<i>T. retusum</i>	2, 7	1	EC 402150
<i>T. campestre</i>	2, 5	1	EC 425028
	2, 4, 5	1	EC 425026
<i>T. argutum</i>	2, 4, 5	1	EC 402154
<i>T. incarnatum</i>	2, 4, 5, 7	2	IG 96-111, EC 402164
<i>T. cherleri</i>	2, 4, 7	1	EC 401703
<i>T. resupinatum</i>	2, 4, 7	12	SH 98-36, SH 98-72, SH 98-73, SH 98-86, JHS-3, SH-99-29, SH-99-65, SH-99-23, SH-99-33, SH-99-32, SH-99-25, SH-99-26
<i>T. angustifolium</i>	1, 4, 7	2	EC 425062, EC 425061
<i>T. medium</i>	1, 4, 7	1	EC 425045
<i>T. constantinopolitana</i>	2, 4, 7	1	EC 401713
<i>T. apertum</i>	2, 4, 7	1	EC 401712
<i>T. glomeratum</i>	2, 4, 7	2	EC 402170, EC 401700

**Plate 12 : Variation for SOD isozyme banding pattern in
different accessions of *Trifolium* species.**

A. From L to R.

T. alexandrinum

JHB 94P-22, JHB 94-31, JHB 94-25, IL 40014, IL 40013,
JHB 94P-60, BL 144, JHB 94-56, BL 142, Raj 7/13-25,
HFB 155

B. From L to R.

T. hybridum [EC 425032], *T. hybridum* [EC 425029],
T. hybridum [EC 425030], *T. echinatum* [EC 401714],
T. echinatum [EC 425014], *T. echinatum* [EC 425076],
T. echinatum [EC 425075], *T. echinatum* [EC 425077],
T. echinatum [EC 425078], *T. alexandrinum* [JHB 94P-22],
T. hybridum [EC 425032]

C. From L to R.

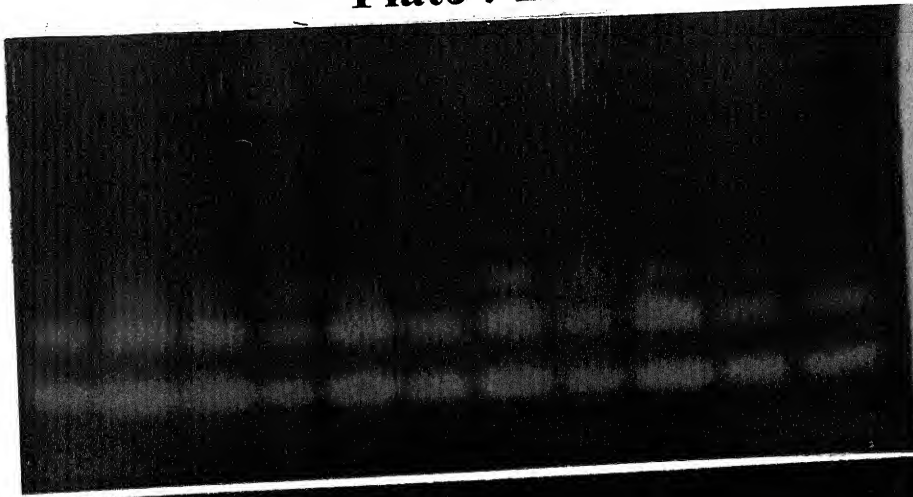
T. alexandrinum [JHB 94P-22], *T. nigrescens* [EC 425049],
T. nigrescens [EC 425047], *T. nigrescens* [EC 425048],
T. campestre [EC 425028], *T. campestre* [EC 425026],
T. hirtum [EC 425039], *T. hirtum* [EC 425037],
T. hirtum [EC 425038], *T. purpureum* [EC 425070],
T. purpureum [EC 425069]

D. From L to R.

T. alexandrinum

EC 329299, IL 40010, EC 400733, Wardan, EC 401711,
EC 401710, EC 401709, EC 402161, EC 400977, EC 400961,
EC 400976

Plate : 12



A



B



C



D

**Plate 13 : Variation for SOD isozyme banding pattern
in different accessions of *Trifolium* species**

A. From L to R.

T. alexandrinum JHB 94P-22, JHB 6/54, JHB 16/2,
JHB 36/5-54, BL 142, IL 40014, IL 40013,
T. nigrescens [EC 425047], *T. campestre* [EC 402155],
Test sample

B. From L to R.

T. alexandrinum JHB 94P-22, Raj 7/53-54, Raj 7/53-54-O,
Raj 7/53-54-2, Raj 7/13-14-O, Raj-Bundi-O, Raj 7/49-50,
Raj 7/53-54, Raj 7/13-25, HFB 155, BL 131.

C. From L to R.

T. alexandrinum JHB 94P-22, Wardan S- 1, Wardan S-2,
Wardan S-3, Wardan S-4, JHB 91P-20, JHB 34/22,
JHB 94-R-35, JHB 94-R-13, Test sample

D. From L to R.

T. alexandrinum [JHB 94P-22], *T. hybridum* [EC 425032],
T. nigrescens [EC 425047], *T. echinatum* [EC 425076],
T. campestre [EC 425026], *T. purpureum* [EC 425070],
T. hirtum [EC 425039], *T. angustifolium* [EC 425061],
T. resupinatum [SH 98-86], *T. alexandrinum* [EC 401711],
T. alexandrinum [JHB 6/54]

Plate : 13



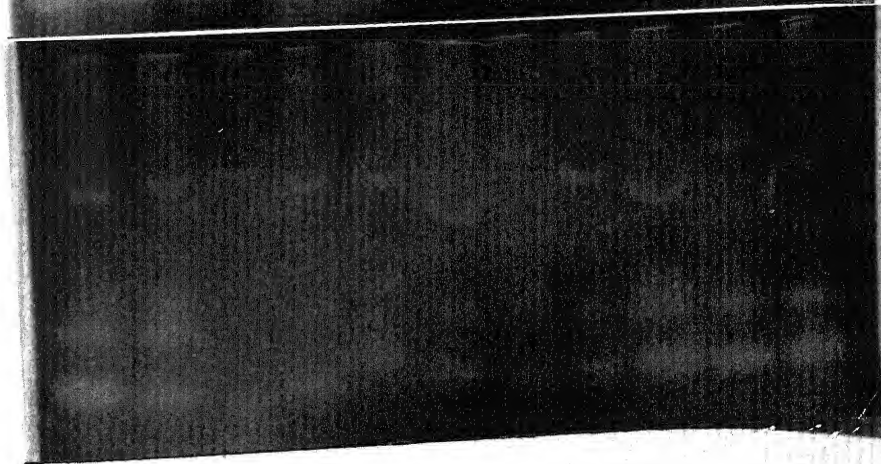
A



B



C



D

Various representatives of *T. alexandrinum* were studied for SOD enzyme system. Presence of three bands i.e. Band 2, 4 and 7 was observed in 52 out of 65 lines (Table 4.16). This group was represented by exotic lines, Rajasthan lines, lines from Punjab and Haryana. Presence of only two bands i.e. 2 and 4 was observed in 11 lines representing red flowered and pentafoliates and progenies. In two accessions i.e. EC 329299 ('Saidi' type) and IL 40010 ('Fahli' type) Bands 2, 4, 5 and 7 were present.

4.3.2.3. Intraspecies variation for Glutamic Oxaloacetic Transaminase (GOT)

Nine accessions of *T. repens* were studied for GOT enzyme variation and band number 9 and 10 were invariably present in all the accessions. All eight accessions of *T. pratense* also did not reveal any variation, and Band 3 and 6 were found common to all (Table 4.17). Five accessions of *T. subterraneum* were studied and Band 4 and 7 were invariably present in all five accessions. Band 2 was also present in four accessions except in EC 402167 which showed presence of Band 3. Thus, total three bands were present in each accession.

Out of total ten bands, Band 8, 9 and 10 were common to all the three accessions of *T. nigrescens*. In three accessions of *T. hirtum* Band 6 was common whereas Band 2 and 5 were present only in EC 425038 and Band 4 present in EC 425039 and EC 425038. Band 7 was present in two accessions namely EC 425037 and EC 425039. Both the accessions of *T. alpestre* possessed Band 1, 6, 7 and 8.

T. hybridum, *T. angustifolium*, *T. purpureum* and *T. resupinatum* did not show any intraspecies variation for GOT and Band 7 & 8 were present in all the accessions of these species. *T. echinatum* (EC 425076) was marked for absence of any GOT band whereas its other accession possessed Band 7 & 8.

In *T. campestre* Band 1, 2, 5 and 7 were present in both the accessions. Four bands 1, 2, 6 and 7 were invariably present in both the accessions of *T. incarnatum*. Single accession of *T. cherleri* showed presence of Band 3, 6 and 7. *T. lappaceum* was noticed for only band i.e. Band 6. *T. diffusum* (EC 402163) was observed for four bands i.e. Band 4, 5, 6 and 7.

In *T. medium* (EC 425045) three bands i.e. Band 3, 6 and 7 were present. *T. constantinopolitanum* (EC 401713) showed presence of Band 1, 6 and 7. Band 6 and

Table 4.16. Zymogram pattern for SOD among different genotypes of *T. alexandrinum*

Enzyme	Bands	No. Genotypes	Genotypes
SOD	2, 4, 7	52	EC 400733, EC 401710, EC 401709, Wardan, EC 402161, EC 400977, EC 400976, EC 401711, EC 318951, JB 92-1, BL 122, JHB 146, Raj 7/49-50, JHTB 9-90 N1, JHB 5-13/12, IL 4009, JHTB 5-90-2, JHTB 3-90-H, JHTB 13-90-B, Raj 7/ 53-54, JHTB-1-90-P3, Raj 7/53-54-O, Raj 7/53-54-2, Raj 7/13-14-O, JHTB 5-90-I, IL 40010-Mes, JHB 94-18/11, JHB 91 P20, JHB 34/22, Raj-Bundi-O, JHB -P-23/35, JHTB -1-90-A1, JHB 94-31, JHB94-25, IL 40014, IL 40013, JHB 94-P-60, BL 144, JHB 94-56, BL 142, Raj 7/13-25, HFB 155, BL 131, JHB 36/5-54, JHB CT2 6/35, JHB 6/54, JHB 16/2, HFB 155, Wardan S-1, Wardan S-2, Wardan S-3, Wardan S-4
	2, 4, 5, 7	2	EC 329299, IL 40010
	2, 4	11	JHB 94P-22, JHB94-R-16, JHB94-R-35, JHB94-R-13, JHB94-R-25, JHB94P/T-34, JHB 57P3, JHB P17-1, Raj 7/13-14, JHB 15-27, JHB 6/54 p/t

Table 4.17. Zymogram pattern for GOT among different accessions of *Trifolium* species

Species	Bands	No. Accession	Accessions
<i>T. repens</i>	9, 10	9	EC401707, EC401704, EC401706, EC400985, EC400986, EC400984, EC400987, EC401708, EC401705
<i>T. pratense</i>	3, 6	8	EC 400979, PRC-3, EC 400980, EC 400982, EC 401721, EC401719, EC 401720, EC 400735
<i>T. subterraneum</i>	2, 4, 7	4	IG 96-112, IG 96-113, EC 401717, EC 401718
	3, 4, 7	1	EC402167
<i>T. nigrescens</i>	8, 9, 10	3	EC 425048, EC 425047, EC 425049
<i>T. hirtum</i>	6, 7	1	EC 425037
	4, 6, 7	1	EC 425039
	2, 4, 5, 6	1	EC 425038
<i>T. diffusum</i>	4, 5, 6, 7	1	EC 402163
<i>T. hybridum</i>	7, 8	5	EC 425032, EC425029, EC425030, EC425029, EC425030
<i>T. alpestre</i>	1, 6, 7, 8	2	EC 425042, EC 425043
<i>T. tembense</i>	2, 3, 5, 6, 7	1	EC 402169
<i>T. echinatum</i>	7, 8	1	EC 401714
	-	1	EC 425076
<i>T. spumosum</i>	2, 3, 6, 7, 8	1	EC 402160
<i>T. lappaceum</i>	6	1	EC 402165
<i>T. purpureum</i>	7, 8	2	EC 425069, EC 425070
<i>T. retusum</i>	3, 7, 8	1	EC 402150
<i>T. campestre</i>	1, 2, 5, 7	2	EC 425028, EC 425026
<i>T. argutum</i>	2, 7, 8	1	EC 402154
<i>T. incarnatum</i>	1, 2, 6, 7	2	IG 96-111, EC 402164
<i>T. cherleri</i>	3, 6, 7	1	EC 401703
<i>T. resupinatum</i>	7, 8	12	SH 98-36, SH 98-72, SH 98-73, SH 98-86, JHS-3, SH-99-29, SH-99-65, SH-99-23, SH-99-33, SH-99-32, SH-99-25, SH-99-26
<i>T. angustifolium</i>	7, 8	2	EC 425062, EC 425061
<i>T. medium</i>	3, 6, 7	1	EC 425045
<i>T. constantinopolitanum</i>	1, 6, 7	1	EC 401713
<i>T. apertum</i>	2, 6, 7	1	EC 401712
<i>T. glomeratum</i>	6, 7	2	EC 402170, EC 401700

**Plate 14 : Variation for GOT isozyme banding pattern in
different accessions of *Trifolium* species.**

A. From L to R.

T. alexandrinum

JHB-P-23/35, JHB 91P-20, JHB 94P-22, JHB 94-R-16,
JHB 94-R-25, JHB 94 P/T-34, EC 318951, JHB 57P3,
JHB P17-1, JHB 94-R-35, JHB 94-R-13

B. From L to R.

T. apertum [EC 401712], *T. constantinopolitanum* [EC 401713],
T. hirtum [EC 402153], *T. diffusum* [EC 402163],
T. tembense [EC 402169], *T. spumosum* [EC 402160],
T. lappaceum [EC 402165], *T. argutum* [EC 402154],
T. retusum [EC 402150], *T. campestre* [EC 402155],
Test sample

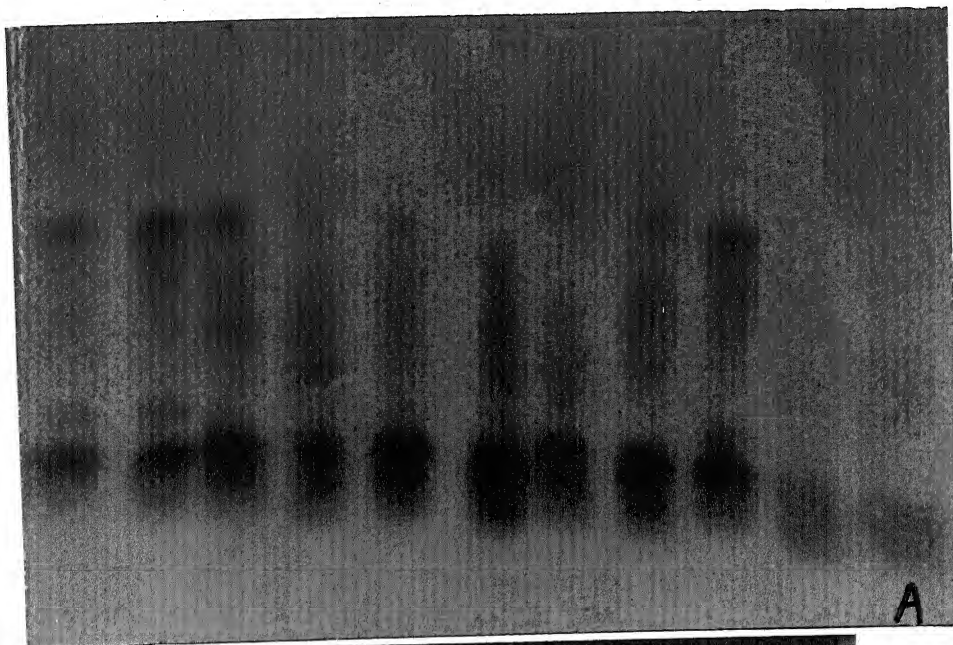
C. From L to R.

T. subterraneum [IG 96-113], *T. subterraneum* [IG 96-112],
T. subterraneum [EC 402167], *T. subterraneum* [EC 401717]
T. subterraneum [EC 402118], Test sample
T. incarnatum [IG 96-111], *T. incarnatum* [EC 402164]
T. cherleri [EC 401703]

D. From L to R.

T. alexandrinum [Wardan], *T. incarnatum* [IG 96-111],
T. pratense [PRC-3], Blank lane ,
T. resupinatum [SH 98-36], *T. resupinatum* [SH 98-72],
T. resupinatum [SH 98-65], *T. incarnatum* [EC 402164]
T. incarnatum [IG 96-111]

Plate : 14



7 were present in both accessions of *T. glomeratum*. The single accession of *T. retusum* (EC 402150) possessed Band 3, 7 and 8. Band 2, 7 and 8 were noticed in *T. argutum* (EC 402154). Five bands viz. Band 2, 3, 6, 7 and 8 were present in *T. spumosum* (EC 402160). *T. tembense* (EC 402169) was also marked for presence of five bands viz. Band 2, 3, 5, 6 and 7. The single accession of *T. apertum* (EC 401712) was studied and three bands i.e. Band 2, 6 and 7 were noticed.

Sixty five lines of *T. alexandrinum* were analysed. Maximum five bands i.e. Band 2, 4, 6, 7, and 8 were present (Table 4.18). Five types of zymogrames were observed in *T. alexandrinum* i.e. (i) 21 lines with Band 2, 4, 6 and 7, (ii) 4 lines with Band 2, 4, 6, 7, and 8, (iii) 4 lines with Band 6, 7 and 8, (iv) 23 lines with Band 2, 6 and 7 and (v) 13 lines with Band 2, 6, 7, and 8. Band 6 and 7 were present in all 5 groups, Band 2 in 4 groups, and Band 8 and 4 present in 3 and 2 groups.

4.3.2.4. Intraspecies variation for Acid Phosphatase (ACP)

Band 1 and 6 were present in all the accessions of *T. repens*, *T. pratense*, *T. purpureum*, *T. angustifolium*, *T. constantinopolitanum*, *T. apertum* and *T. glomeratum* (Table 4.19). All accessions of *T. subterraneum* and *T. hirtum* studied showed presence of Band 1 and 3. Additional Band 4 was present in three accessions of *T. subterraneum* only whereas Band 6 was present only in one accession i.e. EC 401717. Three accessions of *T. nigrescens* one each of *T. campestre* and *T. retusum* and two of *T. alpestre* were marked for presence of only band i.e. Band 1. Band 1 was present in both the accessions of *T. echinatum* in addition to Band 6 in EC 401714. Band 1, 3 and 7 were found common to both the accessions of *T. incarnatum* while Band 8 was present only in IG 96-111. The single accessions each of *T. cherleri* (EC 401703) and *T. lappaceum* (EC 402165) possessed only two bands i.e. Band 1 and 6. Band 1 and 7 were found in single accession of *T. diffusum* (EC 402163). Twelve accessions of *T. resupinatum* were analysed. Most of the accessions possessed three bands. Band 1 was commonly present in all the accessions and Band 5 in ten accessions except two accessions (SH 98-72 and JHS-3). Band 2 and 6 were common in three and five accessions respectively. SH 98-36 was represented with four bands and SH 98-72 with only band. Band 1 and 4 were present in *T. medium*, whereas Band 1 and 2 were present in *T. argutum* EC 402154.

Table 4.18. Zymogram pattern for GOT among different genotypes of *T. alexandrinum*

Enzyme	Bands	No. Genotypes	Genotypes
GOT	2, 4, 6, 7	21	EC 329299, IL 40010, EC 400733, EC 401710, EC 401709, Wardan, EC 402161, EC 400977, EC 400976, EC 401711, JB 92-1, JHB 5-13/12, IL 4009, JHTB 5-90-2, JHTB 3-90-H, JHTB 13-90-B, Raj 7/ 53-54, JHTB-1-90-P3, Raj 7/53-54-O, Raj 7/53-54-2, Raj-Bundi-O.
	2, 4, 6, 7, 8	4	JHB 94P-22, JHBP17-1, JHB91 P-20, JHB-P-23/35.
	2, 6, 7, 8	13	JHB 94-R-16, JHB94-R-25, JHB94P/T-34, EC 318951, JHB 57P3, Raj 7/13-14, BL 122, JHB 146, Raj 7/49-50, JHTB 9-90 N1, Raj 7/13-14-O, IL 40010-Mes, JHTB-1-90-A1.
	6, 7, 8	4	JHB 94-R-35, JHB 94-R-13, JHB 15-27, JHB 6/54 p/t.
	2, 6, 7	23	JHTB 5-90-I, JHB 94-18/11, JHB 34/22, JHB 94-31, JHB94-25, IL 40014, IL 40013, JHB 94-P-60, BL 144, JHB 94-56, BL 142, Raj 7/13-25, HFB 155, BL 131, JHB 36/5-54, JHB CT2 6/35, JHB 6/54, JHB 16/2, HFB 155, Wardan S-1, Wardan S-2, Wardan S-3, Wardan S-4

Table 4.19. Zymogram pattern for ACP among different accessions of *Trifolium* species

Species	Bands	No. Accession	Accessions
<i>T. repens</i>	1, 6	9	EC401707, EC401704, EC401706, EC400985, EC400986, EC400984, EC400987, EC401708, EC401705
<i>T. pratense</i>	1, 6	8	EC 400979, PRC-3, EC 400980, EC 400982, EC 401721, EC401719, EC 401720, EC 400735
<i>T. subterraneum</i>	1, 3, 4	3	IG 96-112, IG 96-113, EC 401718
	1, 3	1	EC 402167
	1, 3, 6	1	EC 401717
<i>T. nigrescens</i>	1	3	EC 425048, EC425037, EC425049
<i>T. hirtum</i>	1, 3	3	EC 425038, EC 425037, EC 425039
	1, 3, 6	1	EC 402153
<i>T. diffusum</i>	1, 7	1	EC 402163
<i>T. hybridum</i>	1	5	EC 425032, EC425029, EC425030, EC425029, EC425030
<i>T. alpestre</i>	1	2	EC 425042, EC425043
<i>T. tembense</i>	1, 2, 6	1	EC 402169
<i>T. echinatum</i>	1, 6	1	EC 401714
	1	1	EC 425076
<i>T. spumosum</i>	1, 3	1	EC 402160
<i>T. lappaceum</i>	1, 6	1	EC 402165
<i>T. purpureum</i>	1, 6	2	EC 425069, EC 425070
<i>T. retusum</i>	1	1	EC 402150
<i>T. campestre</i>	1	2	EC 425028, EC 425026
<i>T. argutum</i>	1, 2	1	EC 402154
<i>T. incarnatum</i>	1, 3, 7, 8	1	IG 96-111
	1, 3, 7	1	EC 402164
<i>T. cherleri</i>	1, 6	1	EC 401703
<i>T. resupinatum</i>	1, 2, 5, 6	1	SH 98-36
	1	1	SH 98-72
	1, 5, 6	3	SH 98-73, SH-99-69, SH-99-32
	1, 5	5	SH 98-86, SH-99-29, SH-99-33, SH-99-25, SH-99-26
	1, 2, 6,	1	JHS-3
	1, 2, 5	1	SH-99-23
<i>T. angustifolium</i>	1, 6	2	EC 425062, EC 425061
<i>T. medium</i>	1, 4	1	EC 425045
<i>T. constantinopolitanum</i>	1, 6	1	EC 401713
<i>T. apertum</i>	1, 6	1	EC 401712
<i>T. glomeratum</i>	1, 6	2	EC 402170, EC 401700

Plate 15 : Variation for isozyme banding pattern in *Trifolium*.

A. From L to R.

T. alexandrinum.

BL 122, JHB 146, Raj 7/ 49-50, JHTB 9-90N1, JHB 5-13/12, IL 4009,
JHTB 5-90-2, JHTB 3-90H, JHTB 13-90B, Raj 7/53-54, JHTB-1-90-P3

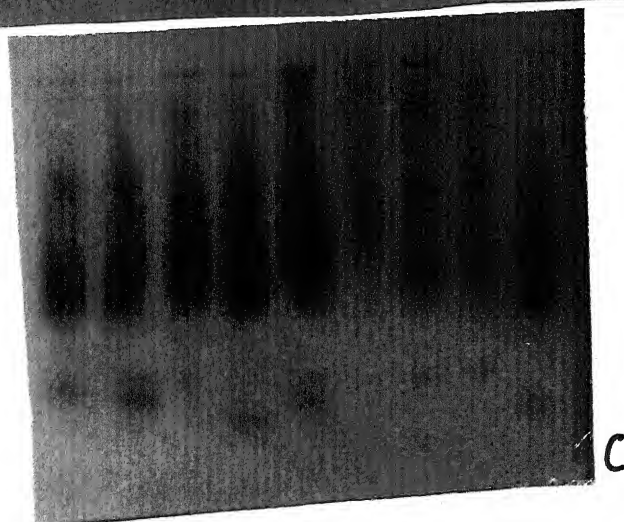
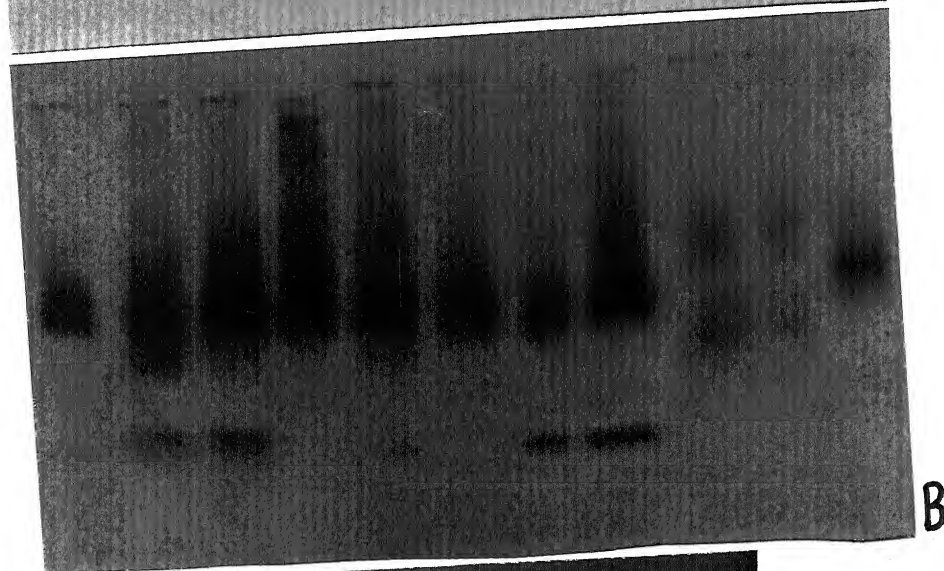
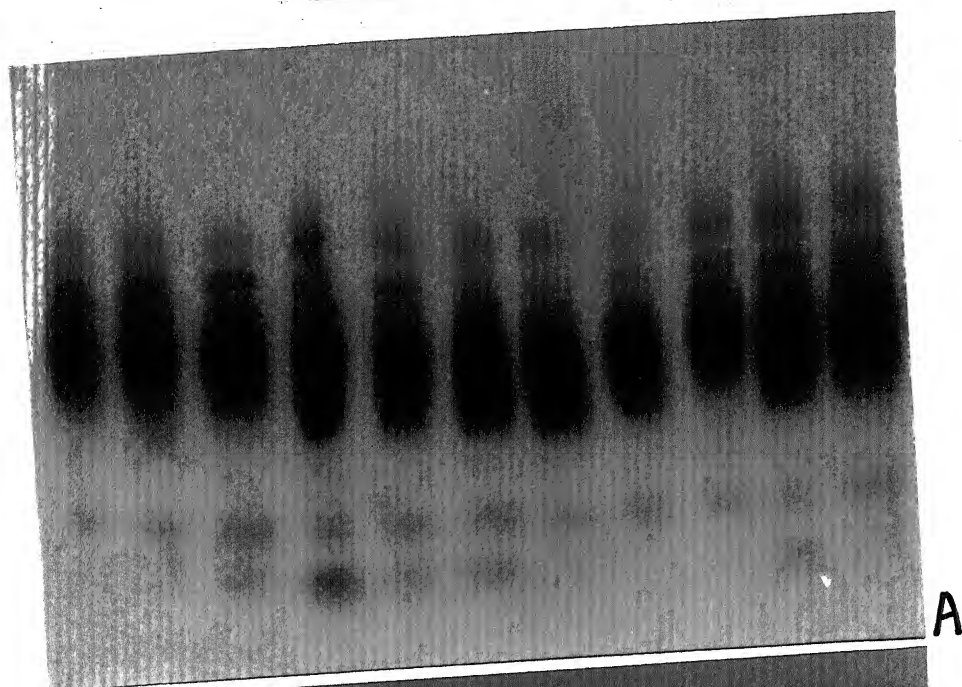
B. From L to R.

<i>T. alexandrinum</i> [JHB 94P-22],	<i>T. resupinatum</i> [SH 98-86],
<i>T. resupinatum</i> [SH 98-73],	<i>T. resupinatum</i> [SH 98-72],
<i>T. resupinatum</i> [SH 98-36],	<i>T. glomeratum</i> [EC 402170],
<i>T. angustifolium</i> [EC 425061],	<i>T. angustifolium</i> [EC 425062],
Test sample	Test sample
<i>T. medium</i> [EC 425045]	

C. From L to R.

<i>T. subterraneum</i> [IG96-113],	<i>T. subterraneum</i> [IG 96-112],
<i>T. subterraneum</i> [EC 422167],	<i>T. subterraneum</i> [EC 401717],
<i>T. subterraneum</i> [EC 401718],	Test sample
<i>T. incarnatum</i> [IG96-111],	<i>T. incarnatum</i> [EC 402164],
<i>T. cherleri</i> [EC 401703]	

Plate : 15



Among sixty five cultivars of *T.alexandrinum* analyzed, Band 1 and 6 were invariably present in all (Table 4.20). Maximum 3 bands *i.e.* Band 1, 4 and 6 were present in 34 lines. Only two bands *i.e.* Band 1 and 6 were present in 31 lines.

4.3.2.5. Intraspecies variation for Peroxidase enzyme

Among nine accessions of *T. repens* analysed for peroxidase, total 10 bands were present. Band A11 and C2 were invariably present in all accessions (Table 4.21). The second highest frequent band was C4 and present in eight accessions. The highest number of 9 bands (4 anodal and 5 cathodal) were present in EC 401707 and the least 5 (2 anodal and 3 cathodal) in two accessions *i.e.* EC 400984 and EC 400987.

Total 6 bands (*i.e.* A-8, A-9, A-11, C-2, C-7 and C-9) were present in all 8 accessions of *T. pratense*. In *T. subterraneum* total bands present were eight (4 anodal+4 cathodal). The highest number of seven bands were present in three accessions and least five bands were present in EC 402167. Bands A-11, C-4 and C-7 were commonly present in all the accessions. Bands A-3, A-8 and C-2 were present in four accessions except EC402167.

Three accessions of *T. nigrescens* were analysed for peroxidase enzyme. Total number of 7 bands (3 anodal + 4 cathodal) were observed. The highest number of five bands were in EC 425047. Band A-7, A-11 and C-11 were found common in 2 accessions *viz.* EC 425048 and EC 425049. Band A-11 was present in all three accessions of *T. nigrescens*. Band A-5, C-4, C-7 and C-9 were found only in EC 425047. Three accessions of *T. hirtum* were studied. A total of 7 bands were represented in different accessions. The highest number in any accession was observed to be seven present in EC 425039 followed with six in EC 425037 and EC 425038. Band A5, A7, A11 and C2 were invariably present in all the accessions whereas Band A4 was present in EC 425038 and EC 425039.

Band A7, A11, C2 and C9 were common to both accessions of *T.alpestre*. Band A7, A11, C2, C4 and C7 were present in both the accessions *T. echinatum*. In addition to these, Band C9 was present in EC 401714.

In *T. purpureum* total 7 bands namely band A5, A7, A11, C2, C4, C5 and C6 were present in both the accessions. Band A10, A11 and C4 were found common

Table 4.20. Zymogram pattern for ACP among different genotypes of *T. alexandrinum*

Enzyme	Bands	No. Genotypes	Genotypes
ACP	1, 4, 6	34	EC 329299, IL 40010, EC 400733, EC 401710, EC 401709, Wardan, EC 402161, EC 400977, EC 400976, EC 401711, JB92-1, BL 122, JHB 146, Raj 7/49-50, JHTB 9-90 N1, JHB 5-13/12, IL 4009, JHTB 5-90-2, JHTB 3-90-H, JHTB 13-90-B, Raj 7/ 53-54, JHTB-1-90-P3, Raj 7/53-54-O, Raj 7/53-54-2, Raj Bundi -O, BL 131, JHB 36/5-54, JHB CT2 6/35, JHB 6/54, JHB 16/2, Wardan S-1, Wardan S-2, Wardan S-3, Wardan S-4
	1, 6	31	JHB 94P-22, JHB94-R-16, JHB94-R-35, JHB94-R-13, JHB94-R-25, JHB94P/T-34, EC 318951, JHB 57P3, JHB P17-1, Raj 7/13-14, JHB 15-27, JHB 6/54 p/t, Raj 7/13-14-O, JHTB 5-90-I, IL 40010-Mes, JHB 94-18/11, JHB 91 P20, JHB 34/22, JHB -P-23/35, JHTB -1-90-A1, JHB 94-31, JHB94-25, IL 40014, IL 40013, JHB 94-P-60, BL 144, JHB 94-56, BL 142, Raj 7/13-25, HFB 155, HFB 155,

Table 4.21. Zymogram pattern for Peroxidase in accessions of *Trifolium* species

Species	Bands		No. of Accession	Accessions
	Anodal	Cathodal		
<i>T. repens</i>	1, 5, 7, 11	2, 4, 7, 8, 11	1	EC 401707
	7, 11	2, 4, 7, 11	1	EC 401704
	1, 5, 11	2, 10, 11	1	EC 401708
	1, 5, 11	2, 4, 10, 11	1	EC 401705
	1, 7, 11	2, 4, 7, 11	1	EC 401706
	1, 7, 11	2, 4, 7, 8, 10	2	EC 400985, EC 400986
	5, 11	2, 4, 8	2	EC 400984, EC 400987
<i>T. pratense</i>	8, 9, 11	2, 7, 9	8	EC 400979, PRC-3, EC 400980, EC 400982, EC 401721, EC 401719, EC 401720, EC 400735
<i>T. subterraneum</i>	3, 7, 8, 11	2, 4, 7	2	IG 96-112, IG 98-113
	7, 11	4, 7, 9	1	EC 402167
	3, 8, 11	2, 4, 7	1	EC 401717
	3, 8, 11	2, 4, 7, 9	1	EC 401718
<i>T. nigrescens</i>	7, 11	11	2	EC 425048, EC 425049
	5, 11	4, 7, 9	1	EC 425047
<i>T. hirtum</i>	4, 5, 7, 11	2, 9	1	EC 425038
	5, 7, 11	2, 9, 11	1	EC 425037
	4, 5, 7, 11	2, 9, 11	1	EC 425039
<i>T. diffusum</i>	6, 7, 11	7	1	EC 402163
<i>T. hybridum</i>	7, 11	1, 2	2	EC 401702, EC 401701
	7, 11	1, 2, 3, 9	1	EC 425032
	7, 11	2, 3, 9	2	EC 425029, EC 425030
<i>T. alpestre</i>	7, 11	2, 9	2	EC 425042, EC 425043
<i>T. tembense</i>	7, 11	4, 5	1	EC 402169
<i>T. echinatum</i>	7, 11	2, 4, 7, 9	1	EC 401714
	7, 11	2, 4, 7	1	EC 425076
<i>T. spumosum</i>	11	4, 7, 9	1	EC 402160
<i>T. lappaceum</i>	7, 11	2, 4, 11	1	EC 402165
<i>T. purpureum</i>	5, 7, 11	2, 4, 5, 6	2	EC 425069, EC 425070
<i>T. retusum</i>	3, 11	2, 5, 7	1	EC 402150
<i>T. campestre</i>	10, 11	4, 11	1	EC 425028
	10, 11	4	1	EC 425026
<i>T. argutum</i>	5, 11	5, 9, 11	1	EC 402154
<i>T. incarnatum</i>	8, 11	2, 4, 11	2	IG 96-111, EC 402164
<i>T. cherleri</i>	5, 11	4, 7, 9	1	EC 401703
<i>T. resupinatum</i>	5, 11	5, 9	2	SH 98-36, SH 98-73
	5, 11	9,	1	SH 98-72,
	5, 11	4	1	SH 98-86
	2, 3, 5, 11	2, 4, 7, 9, 11	1	JHS-3
	2, 11	2, 4, 7	7	SH-99-29, SH-99-69, SH-99-23, SH-99-33, SH-99-32, SH-99-25, SH-99-26
<i>T. angustifolium</i>	7, 11	4, 9	2	EC 425062, EC 425061
<i>T. medium</i>	7, 11	4, 7, 11	1	EC 425045
<i>T. constantinopolitanum</i>	7, 11	2, 4, 7	1	EC 401713
<i>T. apertum</i>	5, 7, 11	7, 9	1	EC 401712
<i>T. glomeratum</i>	1, 5, 11	7, 11	2	EC 402170, EC 401700

in both the accessions of *T. campestre* whereas band C11 was present in EC 425028 only. Band A8, A11, C2, C4 and C11 were common to both the accessions of *T. incarnatum*.

Total 5 bands i.e. bands A5, A11, C4, C7 and C9 were present in *T. cherleri*. Band A7, A11, C2, C4 and C11 were present in *T. lappaceum*. In *T. diffusum* total four bands i.e. bands A6, A7, A11 and C7 were observed.

Twelve accessions of *T. resupinatum* were studied. Maximum 9 bands (four anodal and 5 cathodal) were present in JHS-3. Least number of three bands were noticed in SH 98-86 and SH 98-72. Band number A2, C2, C4 and C7 were quite frequently present in many accessions. Band number A5 was present in 5 accessions. Band number A11 was present in all accessions. Band A3 and C11 were unique to accession JHS 3.

Both the accessions *T. angustifolium* i.e. EC 425062 and EC 425061 were represented with 4 bands (A7, A11, C4 and C9). Band A7, A11, C4, C7 and C11 were present in *T. medium* (EC 425045).

Five bands viz. A7, A11, C2, C4 and C7 were present in *T. constantinopolitanum* (EC 401713). Five bands A1, A5, A11, C7 and C11 were present in both the accessions of *T. glomeratum*. In *T. retusum* (EC 402150) five bands (A3, A11, C2, C5 and C7) were present. Five bands i.e. A5, A11, C5, C9 and C11 were noticed in *T. argutum* (EC 402154).

Only 4 bands i.e. A11, C4, C7 and C9 were present in *T. spumosum* (EC 402160). In *T. tembense* (EC 402169) band A7, A11, C4 and C5 were noticed. In *T. apertum* (EC 401712), five bands viz. A5, A7, A11, C7 and C9 were present.

Five accessions of *T. hybridum* were studied. Six bands were present in EC 425032 whereas four bands were present in EC 401702 and EC 401701. Band A7, A11 and C2 were invariably present in all the accessions. Band C1 was present in 3 accessions i.e. EC 401702, EC 401701 and EC 425032. Band C3 and C9 were noticed in EC 405032, EC 425029 and EC 425030.

Sixty five accessions of *T. alexandrinum* were studied for peroxidase enzyme system. In *T. alexandrinum* two anodal bands i.e. A7 and A11 were invariably present in 64 lines whereas in EC 329299 band A5 was present in place of

Plate 16 : Variation for peroxidase isozyme banding pattern in
accessions of *Trifolium* species.

A. From L to R.

T. alexandrinum

JHB 94 P- 22, JHB 94 -R-16, JHB 94 -R-35, JHB 94 -R-13,
JHB 94 15-27, JHB 94 5-13/12, JHS-3, JHB 94 -18/11,
JHB 94 - 31, JHB 94 - 25, JHB 94 P-22

B. From L to R.

T. alexandrinum

JHB 94 P-22, BL 144, JHB 94-56, BL 142, HFB 155, BL 131,
JHB 6/54, JHB 16/2, JHB 34/22, JHTB-1-90-A1, JHB 94-25

C. From L to R.

T. alexandrinum [JHB 94 P-22], *T. hybridum* [EC 425032],
T. nigrescens [EC 425047], *T. echinatum* [EC 424076],
T. campestre [EC 425026], *T. purpurium* [EC 425070]
T. hirtum [EC 425039], *T. angustifolium* [EC 425061]
T. resupinatum [SH 98-86], *T. alexandrinum* [EC 401711]
T. alexandrinum [JHB 6/54]

D. From L to R.

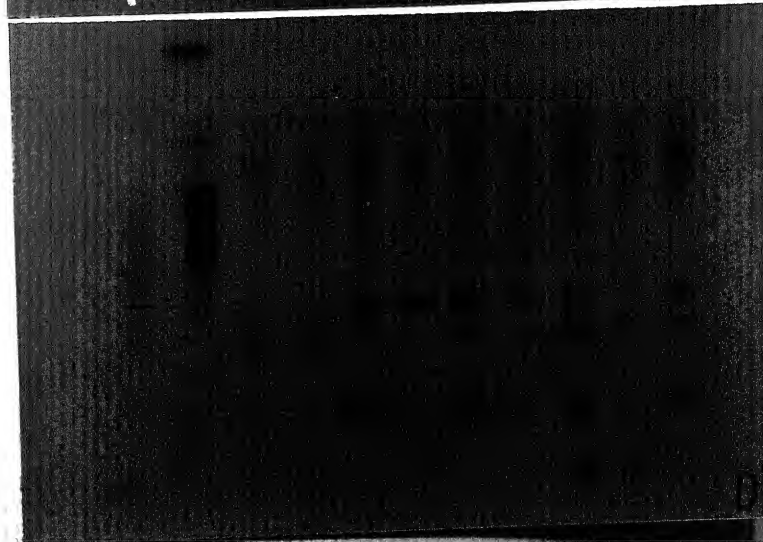
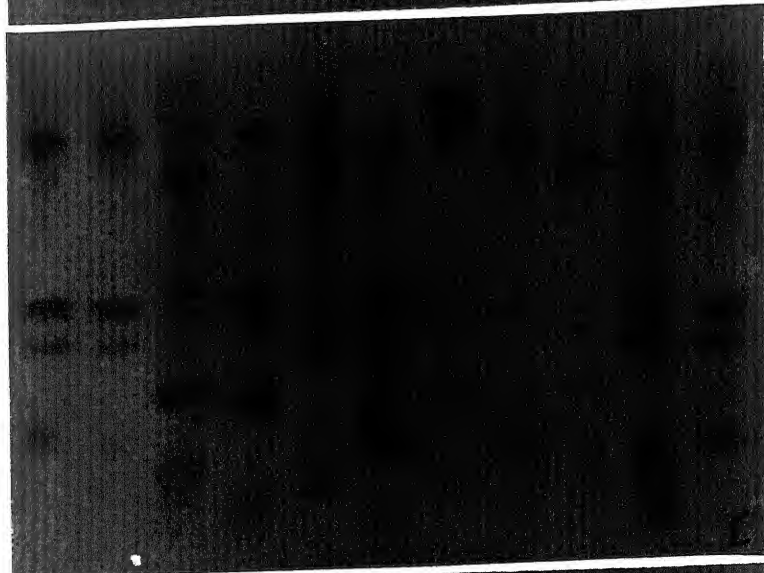
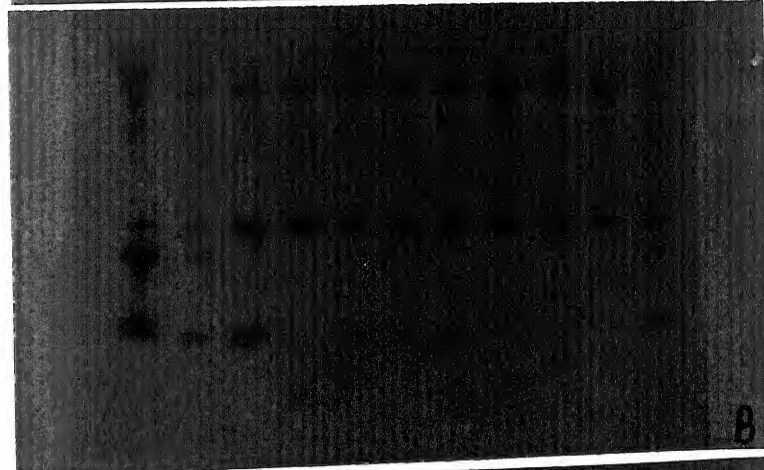
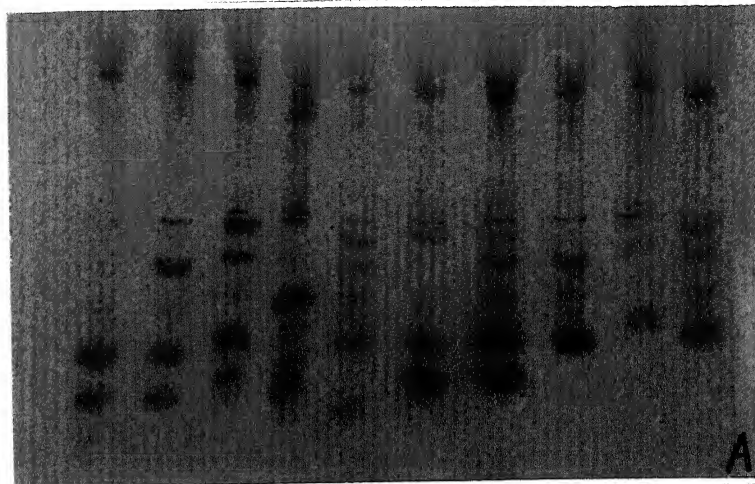
T. alexandrinum

JHB 94P -22, Wardan - S1, Wardan - S2, Wardan - S3, Wardan - S4,
JHB 91 P -20, JHB 34/22, JHB 94 - R - 35, JHB 94 - R- 13 ,

Test sample

Test sample

Plate : 16



**Plate 17 : Variation for peroxidase isozyme banding pattern in
accessions of *Trifolium* species.**

A. From L to R.

Test sample

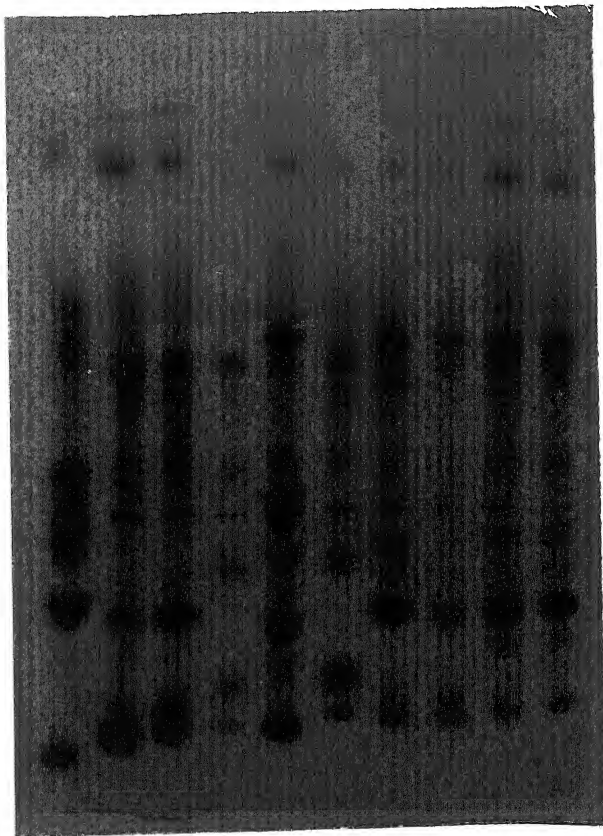
<i>T. glomeratum</i> [EC 402170],	<i>T. glomeratum</i> [EC 401700],
<i>T. repens</i> [EC 401708],	<i>T. repens</i> [EC 401707],
<i>T. repens</i> [EC 401705],	<i>T. repens</i> [EC 401706],
<i>T. repens</i> [EC 401704],	<i>T. repens</i> [EC 400986],
<i>T. repens</i> [EC 400985]	

B. From L to R.

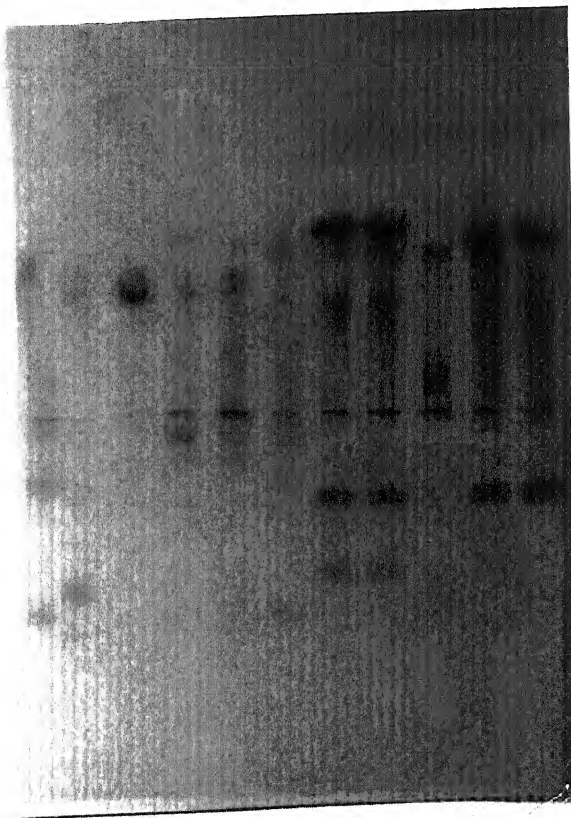
Test sample

<i>T. cherleri</i> [EC 401703],	Test sample
<i>T. incarnatum</i> [IG-96-111],	<i>T. incarnatum</i> [EC 402164],
Test sample	<i>T. subterraneum</i> [IG-96-113],
<i>T. subterraneum</i> [IG-96-112],	<i>T. subterraneum</i> [EC 402167],
<i>T. subterraneum</i> [EC 401717];	<i>T. subterraneum</i> [EC 401718]

Plate : 17



A



B

band A7 (Table 4.22). The maximum number of 5 cathodal bands (i.e. C2, C4, C7, C9, and C11) were present in two accessions i.e. Raj 7/35-54-2 and EC 400977 whereas most of the lines possessed four bands. Band C4 was invariably present in all accessions. The second highest frequency was that of C2 which was present in 64 accessions. Band C7 was found in 62 accessions. Band C9 and C11 were present each in 21 lines. Band C5 was present in EC 329299 (Saidi type) only. Combination of three bands i.e. C2, C4 and C7 were present in 62 accessions. In 18 accessions 4 bands viz-C2, C4, C7 and C11 were present whereas band C2, C4, C7 and C9 were observed in 17 accessions.

4.3.3. Interspecific isozyme variation

4.3.3.1. Interspecific diversity for Esterase

Out of total 18 bands distributed over 25 species, Band 3 was represented in maximum 13 species followed with Band 13 in 12 species (Table 4.23, Fig.10). Six bands were considered as species specific viz. Band 1 in *T. purpureum*, Band 2 in *T. medium*, Band 9 in *T. repens*, Band 15 in *T. incarnatum*, Band 17 in *T. resupinatum* and band 18 in *T. angustifolium*. The highest number of 8 bands were present in *T. resupinatum*. Three species viz. *T. lappaceum*, *T. diffusum* and *T. constantinopolitanum* were represented by a single band.

4.3.3.2. Interspecific diversity for Super-oxide-dismutase isozyme

Out of total 8 bands identified for the genus, Band 4 was found to be most common and was represented in 22 species out of 25 studied (Table 4.24, Fig 11). The band was absent in *T. diffusum*, *T. retusum* and *T. spumosum*. The second highest frequency was that of band 7 which was present in 21 species and absent in *T. hirtum*, *T. echinatum*, *T. campestre* and *T. argutum*. Band 2 was present in 18 species except *T. subterraneum*, *T. diffusum*, *T. angustifolium*, *T. purpureum*, *T. alpestre*, *T. lappaceum* and *T. medium*. Band 5 was represented in only 7 species while band 1 was present in three species i.e. *T. purpureum*, *T. angustifolium* and *T. medium*. Band 3 was uniquely present in two out of three accessions of *T. hirtum*. Band 6 and band 8 were species specific and present in *T. echinatum* and *T. hirtum* respectively.

Table 4.22. Zymogram pattern for Peroxidase among different genotypes of *T. alexandrinum*

Enzyme	Bands		No. Genotypes	Genotypes
	Anodal	Cathodal		
Peroxidase	7, 11	2, 4, 7, 11	18	IL 40010, EC 401711, JHTB 9-90 N1, IL 4009, JHTB 3-90-H, JHTB-1-90-P3, Raj 7/53-54-O, IL 40010-Mes, Raj-Bundi-O, JHB 94-31, IL 40014, BL 131, JHB 36/5-54, JHB CT2 6/35, Wardan S-1, Wardan S-2, Wardan S-3, Wardan S-4
	7, 11	2, 4, 7	25	EC 400733, EC 401710, EC 401709, Wardan, EC 402161, EC 400976, JHB94-R-16, JHB94-R-35, JHB94-R-25, JHB 57P3, JHB P17-1, JHB 15-27, JHB 6/54 p/t, JHB 146, JHB 5-13/12, JHTB 5-90-2, JHTB 13-90-B, Raj 7/ 53-54, JHB 34/22, JHB -P-23/35, JHB94-25, JHB 94-56, JHB 6/54, JHB 16/2, HFB 155
	7, 11	2, 4, 7, 9	17	JHB 94P-22, JHB94-R-13, JHB94P/T-34, Raj 7/13-14, JB92-1, BL 122, Raj 7/49-50, Raj 7/13-14-O, JHTB 5-90-I, JHB 94-18/11, JHB 91 P20, JHTB -1-90-A1, IL 40013, JHB 94-P-60, BL 144, BL 142, HFB 155
	7, 11	2, 4, 9	1	EC 318951
	7, 11	2, 4, 7, 9, 11	2	EC 400977, Raj 7/53-54-2
	7, 11	2, 4, 9, 11	1	Raj 7/13-25
	5, 11	4, 5	1	EC 429299

Table 4.23. Esterase isozyme bands among different species of <i>Trifolium</i>																		
Species	Bands																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
<i>T. repens</i>				+++	+++	+++	++++		++++		++++					++		
<i>T. pratense</i>										++++			++++					
<i>T. cherleri</i>						++				++++			++++					
<i>T. spumosum</i>			+++			++				++++			++++					
<i>T. subterraneum</i>								++					++++	++++		+		
<i>T. resupinatum</i>							++++			++++	++++	++	++	++		++++	+++	
<i>T. alexandrinum</i>			++					+++			++		+++	+++		+		
<i>T. nigrescens</i>			+					++					+	+++				
<i>T. glomeratum</i>			+++									++		+++				
<i>T. apertum</i>			++	++	+++			+++						+++				
<i>T. alpestre</i>			++++										++	+++				
<i>T. hybridum</i>			++++		++		++						++	+++				
<i>T. echinatum</i>			+++				+++				++							
<i>T. purpureum</i>	+		+++	+++				++		++			+			++		
<i>T. angustifolium</i>								++								+		+
<i>T. medium</i>		++	+++		+++					+++								
<i>T. lappaceum</i>																		
<i>T. constantinopolitanum</i>							+++											
<i>T. tembense</i>								+++			++							
<i>T. retusum</i>			+++	++						++						++		
<i>T. hirtum</i>			++		++						+++		+++					
<i>T. campestre</i>			+++		+++			+					+					
<i>T. incarnatum</i>						++	++			+		+++		++	++			
<i>T. arvense</i>				+						+++		+++		++				
<i>T. diffusum</i>										+++								
+ = Faint band									+++ = Medium dark band					++++ = Dark band				

Table 4.24. SOD isozyme bands among different species of <i>Trifolium</i>								
Species	Bands							
	1	2	3	4	5	6	7	8
<i>T. repens</i>		++		+++	++++		+++	
<i>T. pratense</i>		++		+++			+++	
<i>T. cherleri</i>		++		+++			+++	
<i>T. spumosum</i>		+++		+++			+++	
<i>T. subterraneum</i>		++		++			+++	
<i>T. resupinatum</i>		++		+++	++++		+++	
<i>T. alexandrinum</i>		+		+++			+++	
<i>T. nigrescens</i>		++		+++			+++	
<i>T. glomeratum</i>		++		+++			+++	
<i>T. apertum</i>		++		+++			+++	
<i>T. alpestre</i>		+++		+++		+++		
<i>T. hybridum</i>		++		+++	+++		++	
<i>T. echinatum</i>	++			+++			+++	
<i>T. purpureum</i>	++			+++			+++	
<i>T. angustifolium</i>	+++			+++			+++	
<i>T. medium</i>				+++			+++	
<i>T. lappaceum</i>		++		+++			+++	
<i>T. constantinopolitanum</i>		++						+++
<i>T. temense</i>		++						
<i>T. retusum</i>		++	+++	+++	+++			
<i>T. hirtum</i>		++		+++	+++		+++	
<i>T. campestre</i>		++		+++	+++			
<i>T. incarnatum</i>		+++		+++	+++		+++	
<i>T. argutum</i>					++			
<i>T. diffusum</i>							++++ = Dark band	
++ = Faint band		++ = Light band	+++ = Medium dark band					

4.3.3.3. Interspecific diversity for Glutamate-oxalo-acetate transaminase (GOT)

Total 10 bands distributed over 25 species were identified. One to five bands were present in various species (Table 4.25, Fig. 12). Of these, Band 7 was most common and present in 21 species and absent in *T. repens*, *T. pratense*, *T. nigrescens* and *T. lappaceum*. Band 6 present in 14 species was second most common band. Band 4 and 5 were present in four species each. Band 4 was present in *T. subterraneum*, *T. alexandrinum*, *T. hirtum* and *T. diffusum*. Band 1 was present in two species i.e. *T. campestre* and *T. incarnatum*. Band 5 was represented in four species namely *T. hirtum*, *T. campestre*, *T. diffusum* and *T. tembense*. The maximum of five bands were present in four species i.e. *T. hirtum*, *T. alexandrinum*, *T. spumosum* and *T. tembense*. The species with four bands were *T. subterraneum*, *T. campestre*, *T. incarnatum*, *T. diffusum* and *T. alpestre*. The species represented by three bands only were *T. nigrescens*, *T. medium*, *T. constantinopolitanum*, *T. retusum*, *T. argutum* and *T. apertum*. *T. lappaceum* showed presence of only band i.e. Band 6. Band 9 and 10 were recorded in only two species i.e. *T. repens* and *T. nigrescens*.

4.3.3.4. Interspecific diversity for Acid Phosphatase (ACP)

Based on relative mobility of bands, a total of eight bands were found to be distributed throughout the genus. The slowest band no.1 was represented in all the 25 species studied, hence, was identified as genus specific band. The intensity of this band was also very high. (Table 4.26, Fig. 13) The second highest frequency was that of Band 6 which was present in 15 species. Band 3 was present in four species (*T. subterraneum*, *T. hirtum*, *T. incarnatum* and *T. spumosum*). Band 2 was present in three species viz. *T. resupinatum*, *T. argutum* and *T. tembense*. Band 4 was present in *T. subterraneum*, *T. medium* and *T. alexandrinum*. Band 7 was present only in two species i.e. *T. incarnatum* and *T. diffusum*. Band 5 and 8 were found to be species specific bands. Band 5 was specific to *T. resupinatum* and Band 8 specific to *T. incarnatum*. A total of 14 species possessed only two bands whereas five species showed presence of one band only. Three species each were having three and four bands.

Table 4.25. GOT isozyme bands among different species of <i>Trifolium</i>										
Species	Bands									
	1	2	3	4	5	6	7	8	9	10
<i>T. repens</i>						+++			++++	
<i>T. pratense</i>			++++			++++	++			
<i>T. cherleri</i>			++++			++++	++++	++		
<i>T. spumosum</i>		++	++	++++			++++			
<i>T. subterraneum</i>		++++	+++				++	++		
<i>T. resupinatum</i>		++++		+++		+++	++++	+++		
<i>T. alexandrinum</i>		++++					++	++	+++	+++
<i>T. nigrescens</i>						++	++			
<i>T. glomeratum</i>						++	++			
<i>T. apertum</i>		++		+++		+++	++	++		
<i>T. alpestre</i>		++					++	++		
<i>T. hybridum</i>							++	++		
<i>T. echinatum</i>							++	++		
<i>T. purpureum</i>							++	++		
<i>T. angustifolium</i>							++			
<i>T. medium</i>			++			+++				
<i>T. lappaceum</i>						+++				
<i>T. constantinopolitanum</i>		++				++	++			
<i>T. temense</i>		+++	++		+++	+++	+++	+++		
<i>T. reusum</i>		++	+++			++	++			
<i>T. hirtum</i>	+++	+++		++	+++	++	+++			
<i>T. campestre</i>	++	+++			+++	+++	+++			
<i>T. incarnatum</i>		+++				+++	+++	+++		
<i>T. argutum</i>		++		++		++	++	++		
<i>T. diffusum</i>				++	++	++	++			
+ = Faint band	++ = Light band		+++ = Medium dark band				++++ = Dark band			

4.3.3.5. Interspecific diversity for Peroxidase enzyme

Eleven bands each at anodal and cathodal ends were identified based on their relative mobility. These were distributed in different species in various combination (Table 4.27, 4.28, Fig. 14). Band A11 was invariably present in all 25 species, this was identified as genus specific. The second highest frequency was that of band A7 (present in 16 species) followed with band A5 which was present in 10 species. Band A1 was present only in two species i.e. *T. repens* and *T. glomeratum* while band A3 in three species i.e. *T. subterraneum*, *T. resupinatum* and *T. retusum*. Band A8 was also present in only 3 species namely *T. pratense*, *T. subterraneum* and *T. incarnatum*. Some bands were found species specific such as band A2 in *T. resupinatum*, band A4 in *T. hirtum*, band A9 in *T. pratense*, band A10 in *T. campestre* and band A6 in *T. diffusum*. The highest number of four anodal bands were present in *T. repens*, *T. subterraneum*, *T. hirtum* and *T. resupinatum*. Most of the species possessed 2 anodal bands. *T. spumosum* was represented with single anodal band.

Among cathodal peroxidase bands the highest frequency was that of band C4 which was present in 16 out of 25 species. Next highest frequency was of band C7 which was present in 15 species followed by band C2 and C9 in 14 species each. Some bands were found in single species and identified as species specific bands such as band C1 in *T. hybridum* band C6 in *T. purpureum*, band C8 and C10 in *T. repens*. The maximum number of bands in any species was six in *T. repens* and *T. resupinatum*. Ten species were represented with 3 bands, six species with 2 bands, five species with 4 bands and *T. diffusum* with single band.

4.3.4. Estimation of intra specific variability

Based on frequency of isozyme bands differing among accessions of a species and number of zymograms observed an estimate of intra specific variability was estimated (table 4.29).

A total of 46 types of zymograms for esterase isozyme pattern were observed in 25 different species of *Trifolium* and this difference was due to the presence or absence of any one or more than one of all the 18 bands. In case of *T. resupinatum* and *T. hybridum* the maximum number 8-9 bands contributed towards the variability

Table 4.27. Anodal Peroxidaes isozyme bands among different species of <i>Trifolium</i>											
Species	Bands	1	2	3	4	5	6	7	8	9	10
<i>T. repens</i>	PER-A	+				+++		++			11
<i>T. pratense</i>									++++		+++
<i>T. cherleri</i>						++++					+++
<i>T. spumosum</i>											+++
<i>T. subterraneum</i>				++++				+++	++++		+++
<i>T. resupinatum</i>			++++	+		++++					+++
<i>T. alexandrinum</i>						++++		++++			+++
<i>T. nigrescens</i>						+++		++			+++
<i>T. glomeratum</i>						++++					+++
<i>T. apertum</i>	+					+		++++			+++
<i>T. alpestre</i>								++++			+++
<i>T. hybridum</i>								++++			+++
<i>T. echinatum</i>								++++			+++
<i>T. purpureum</i>						+		+			+++
<i>T. angustifolium</i>								++++			+++
<i>T. medium</i>								++++			+++
<i>T. lappaceum</i>								++++			+++
<i>T. constantinopolitanum</i>								++++			+++
<i>T. temense</i>								++++			+++
<i>T. retusum</i>			+++								+++
<i>T. hirtum</i>					+++	+++		++++			+++
<i>T. campestre</i>									++++		+++
<i>T. incarnatum</i>						+++					+++
<i>T. argutum</i>							++++				+++
<i>T. diffusum</i>								++++			+++
+	Faint band		++ = Light band	+++ = Medium dark band				++++ = Dark band			

Table 4.28. Cathodal Peroxidaes isozyme bands among different species of <i>Trifolium</i>												
Species	PER-C	1	2	3	4	5	6	7	8	9	10	11
<i>T. repens</i>			+++		+++			+++	+++		++	+++
<i>T. pratense</i>			+++					+++		+++		
<i>T. cherleri</i>					+			+++		+++		
<i>T. spumosum</i>					+++			+++		+++		
<i>T. subterraneum</i>			+++		+++			+++		+++		+++
<i>T. resupinatum</i>			+++		+++	+++		+++		+++		+++
<i>T. alexandrinum</i>			+++		++	++		+++		+++		+
<i>T. nigrescens</i>					+			+++		+		+++
<i>T. glomeratum</i>								+++				+++
<i>T. apertum</i>								+++		+++		
<i>T. alpestre</i>			+++							+++		
<i>T. hybridum</i>	+++	+++	+++	+++						+++		
<i>T. echinatum</i>		+	+		+			+++		+		
<i>T. purpureum</i>		+			+++	+++	+++					
<i>T. angustifolium</i>					+++					+++		+++
<i>T. medium</i>					+			+++				+
<i>T. lappaceum</i>		+	+	+	+							
<i>T. constantinopolitanum</i>		+++	+++		+			+++				
<i>T. temense</i>					+++	+++						
<i>T. retusum</i>			+++			+++		+++				+++
<i>T. hirtum</i>			+++							+++		+++
<i>T. campestre</i>					+++							+
<i>T. incarnatum</i>			+		+	+				+++		+++
<i>T. argutum</i>								+++				
<i>T. diffusum</i>												
+ = Faint band		++ = Light band		+++ = Medium dark band				++++ = Dark band				

whereas in many other cases only 1-2 bands were responsible for intra species zymogram variation. The estimates of variability revealed maximum variation for zymogram pattern in *T. resupinatum* (4.241) followed with 3.01 in *T. nigrescens*. Fourteen species showed no intraspecies variation whereas cumulative estimate of variability revealed high degree of variation for types of zymogram represented in different *Trifolium* species, the variability estimate was recorded as 4.38.

SOD isozyme banding pattern observed in different species of *Trifolium* revealed that 2-4 bands out of total 8, contributed towards variability among six species only whereas 19 species showed no intra-species variation for SOD banding pattern. Estimate of variability also ranged from 0.458 to 2.667. The total estimate of variability across species was also quite less (1.082) as compared to estimate of variability for esterase.

In case of GOT isozyme banding pattern only 2 species showed intra-species variation for zymogram pattern but a total of 26 types of zymograms were noticed among different species accounting for variability estimate of 2.306. All the 10 bands contributed for intra-species variation

Out of total 8 isozyme bands for ACP observed among different *Trifolium* species, Band number 1 was common to all. The remaining 7 bands contributed to an variability estimate of 1.428 in the genus. Sixteen different types of ACP zymograms were noticed. The intraspecies zymogram variation was observed only in 6 species and 1-3 bands were found different among different accessions of same species. Highest variation for intraspecies zymogram pattern was observed in *T. resupinatum* (4.526).

Zymogram pattern based on 11 anodal and 11 cathodal bands of Peroxidase enzyme showed high degree of variation among different species of the genus. Overall estimate of variation was to the tune of 4.833 and 51 types of zymograms were observed among different species. Out of total 22 bands present, one (A11) was common to all and 21 bands accounted for different zymogram pattern. The intraspecies variation for type of zymograms was observed in 9 species. One to nine bands contributed towards intra-species variation for zymogram pattern. The estimate of variation ranged from 0.938- 3.359. The highest variation was recorded in *T. repens* i.e. 3.359.

T. repens, *T. subterraneum*, *T. resupinatum*, *T. echinatum*, *T. hirtum*, *T. campestre* and *T. incarnatum* had high values for variability estimates whereas *T. alexandrinum* showed very little estimate of variation. Except for esterase isozyme, *T. alexandrinum* was noticed for presence of more than one type of zymogram for different enzyme.

Each species possessed its peculiar zymogram for esterase isozyme except one zymogram which was common to *T. constantinopolitanum* and one accession of *T. hybridum*. The presence of number of bands also varied from 1 to 6.

Generally, the interspecies variability for esterase zymogram was higher than the intra species variation and almost all the species had their specific zymogram. Presence of only one type of zymogram among 65 accessions of *T. alexandrinum* shows that the species has no variation for esterase isozyme. The bands of *T. alexandrinum* were found scattered in different accession of *T. resupinatum*. It also showed 2 bands common with *T. apertum*. In all, one or more bands of *T. alexandrinum* were found common with 20 species under study. The four species i.e. *T. argutum*, *T. diffusum*, *T. incarnatum* and *T. constantinopolitanum* possessed no common band with *T. alexandrinum*.

4.3.5 Similarity Among Different Accessions and Species of *Trifolium*

4.3.5.1 Intraspecies similarity

T. repens:

The dendrogram based on five enzyme system of nine accessions of *T. repens* showed the presence of three clusters (Fig 15 & 16). Maximum five accessions were present in cluster No. 2 whereas, cluster number 1 and 3 were represented by two accessions each.

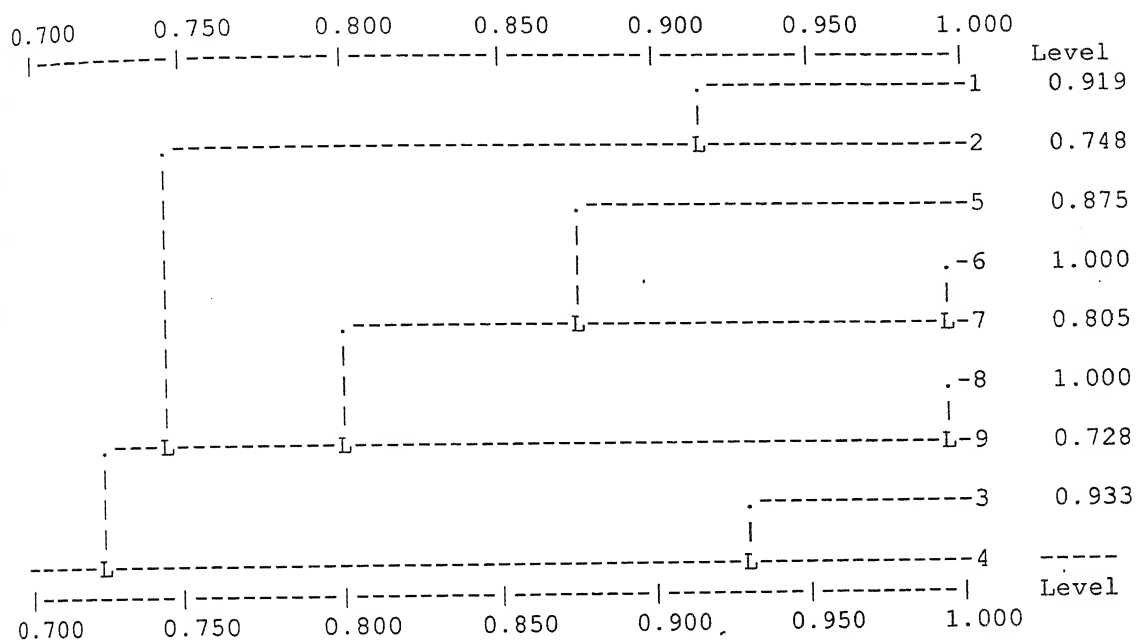
Cluster No.	Accessions
1	EC 401707, EC 401704
2	EC 401706, EC 400985, EC 400986, EC 400984, EC 400984-1
3	EC 401708, EC 401705

In cluster no. 1 EC 401704 was 92% similar with EC 401707 (Table 4.30). Cluster no. 1 showed 75% similarity with cluster number 2 comprising of five

Table 4.30. Similarity matrix based on isozyme banding pattern in *Trifolium repens* accessions

Sr. No.	Accession no.	1	2	3	4	5	6	7	8	9
1	EC 401707	1.00								
2	EC 401704	0.92	1.00							
3	EC 401708	0.69	0.63	1.00						
4	EC 401705	0.74	0.69	0.93	1.00					
5	EC 401706	0.83	0.85	0.71	0.77	1.00				
6	EC 400985	0.78	0.73	0.71	0.77	0.88	1.00			
7	EC 400986	0.78	0.73	0.71	0.77	0.88	1.00	1.00		
8	EC 400984	0.73	0.67	0.71	0.79	0.76	0.83	0.83	1.00	
9	EC 400987	0.73	0.67	0.71	0.79	0.76	0.83	0.83	1.00	1.00

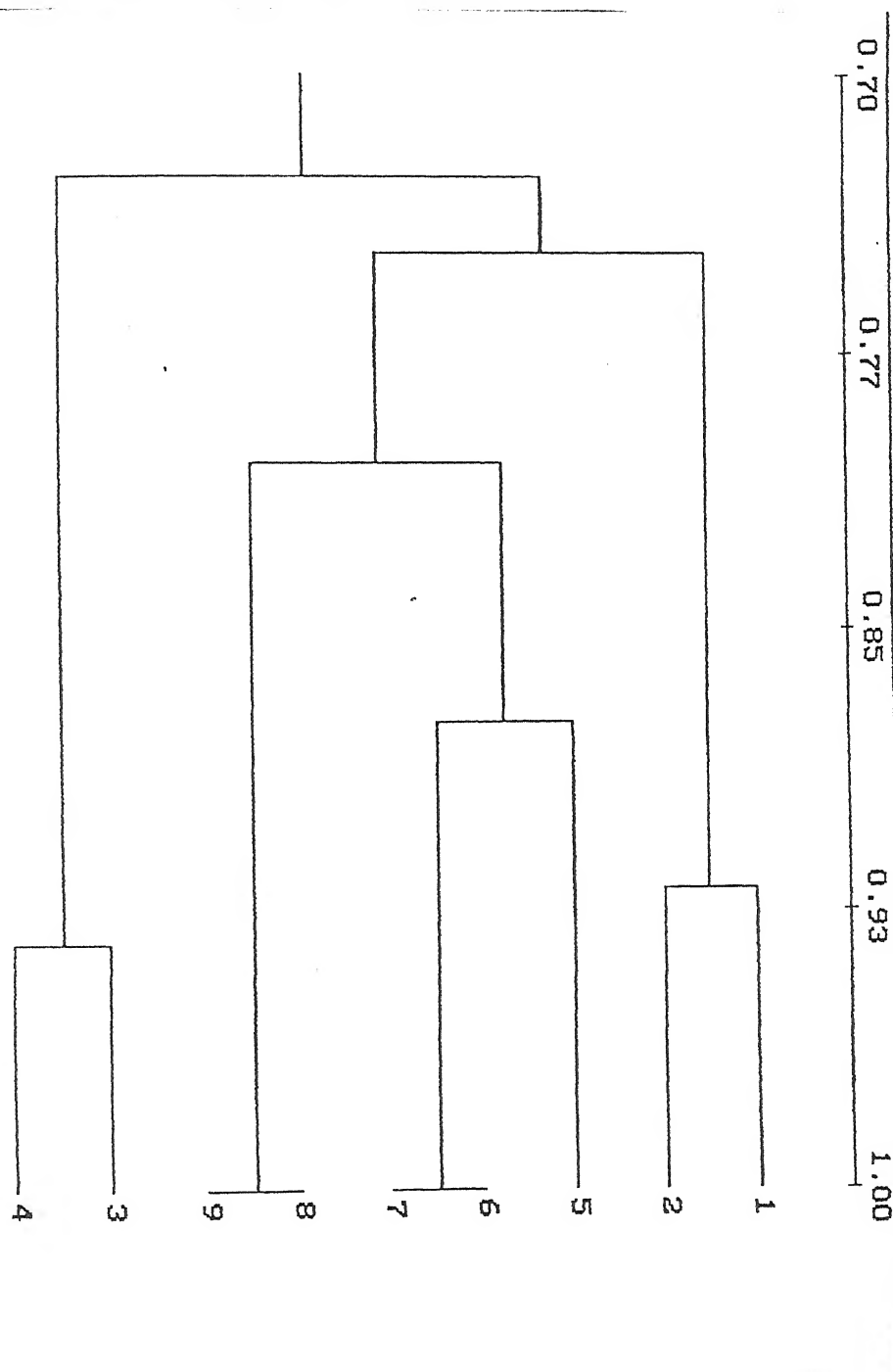
Fig 15. Clustering based on isozyme data showing genetic relatedness among accessions of *Trifolium repens*.



List of accessions

1. EC 401707 2. EC 401704 3. EC 401708 4. EC 401705 5. EC 401706
6. EC 400985 7. EC 400986 8. EC 400984 9. EC 400987

Fig 16 : Dendrogram based on cluster analysis of isozyme data showing genetic relatedness between *T. repens* accessions.



T. repens accessions

1. EC 401707 2. EC 401704 3. EC 401708 4. EC 401705 5. EC 401706
6. EC 400985 7. EC 400986 8. EC 400984 9. EC 400987

accessions. In cluster no. 2, EC 400986 was 100% similar to EC 400985 and showed 87% similarity with EC 401706. Eighty percent similarity was observed between EC 400984 with other three accessions (EC 400986, EC 400985 and EC 401706). In cluster no. 3, 93% similarity was observed between EC 401705 and EC 401708 and cluster 3 showed 73% similarity with other clusters.

T. resupinatum:

Dendrogram obtained on the basis of five enzyme system of 12 accessions of *T. resupinatum* showed the presence of five clusters (Fig.17,18). Maximum five accessions were present in cluster number 5, whereas three accessions were present in cluster number 1. Cluster no. 2 and 3 each were represented with single accessions *i.e.* SH 98-72 and JHS-3 respectively.

Cluster No.	Accessions
1	SH 98-36, SH 98-73, SH 98-86
2	SH 98-72
3	JHS-3
4	SH-99-29, SH-99-25
5	SH-99-69, SH-99-33, SH-99-23, SH-99-32, SH-99-26

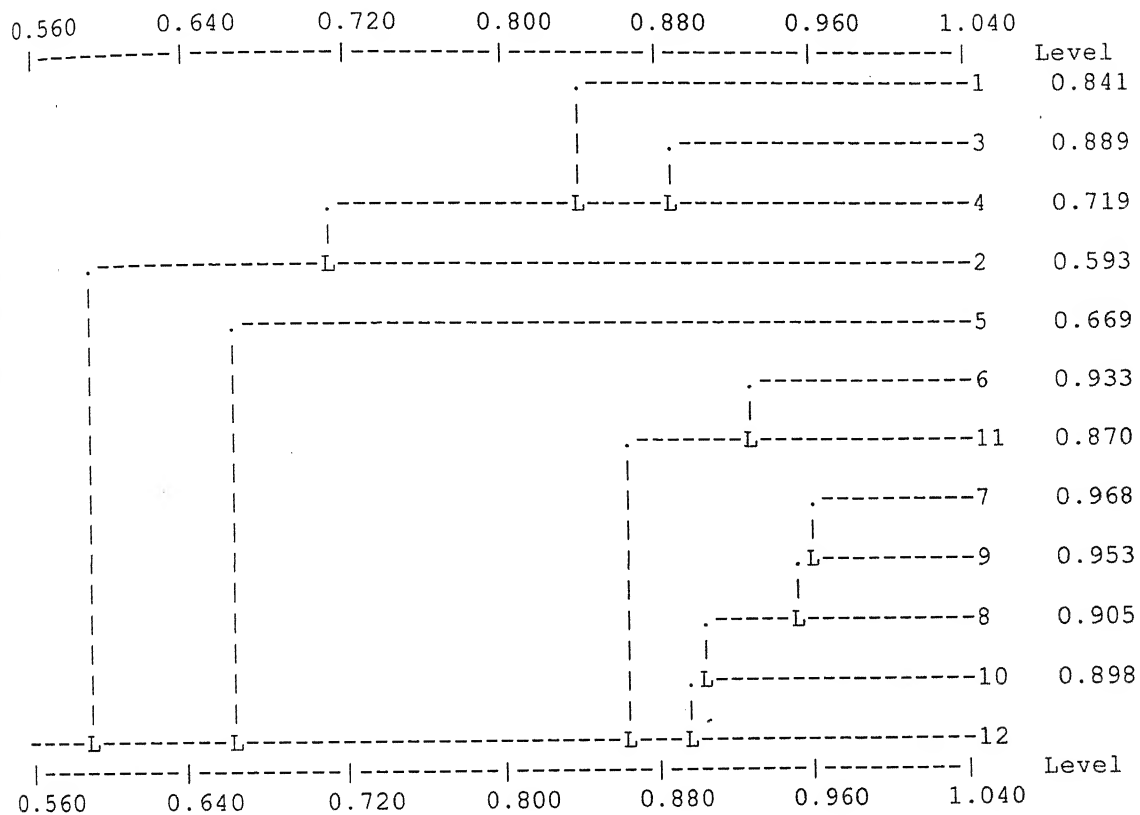
In cluster no. 1, SH 98-86 showed 89% similarity with SH 98-73 (Table 4.31) and both were observed for 84% similarity with SH 98-36. Cluster number 2 was 72% similar to cluster no. 1. JHS-3 made separate cluster *i.e.* cluster no. 3 which showed 67% similarity with cluster no. 4 and 5.

In cluster 4, SH-99-25 was 93% similar with SH-99-29. In cluster 5, SH-99-33 showed 97% similarity with SH-99-69 whereas SH-99-23 was 95% similar with these two. SH-99-32 showed 91% similarity with group of SH-99-69, SH-99-33 and SH-99-23 accessions. SH-99-26 showed 90% similarity with rest of the accessions of cluster no. 5.

T. pratense

Dendrogram obtained on the basis of five enzyme system of 8 accessions of *T. pratense* showed the presence of 2 clusters. 100% within group similarity was found in both the clusters (Table 4.32). Cluster number 1 showed 96.6% similarity with cluster number 2 (Fig.19).

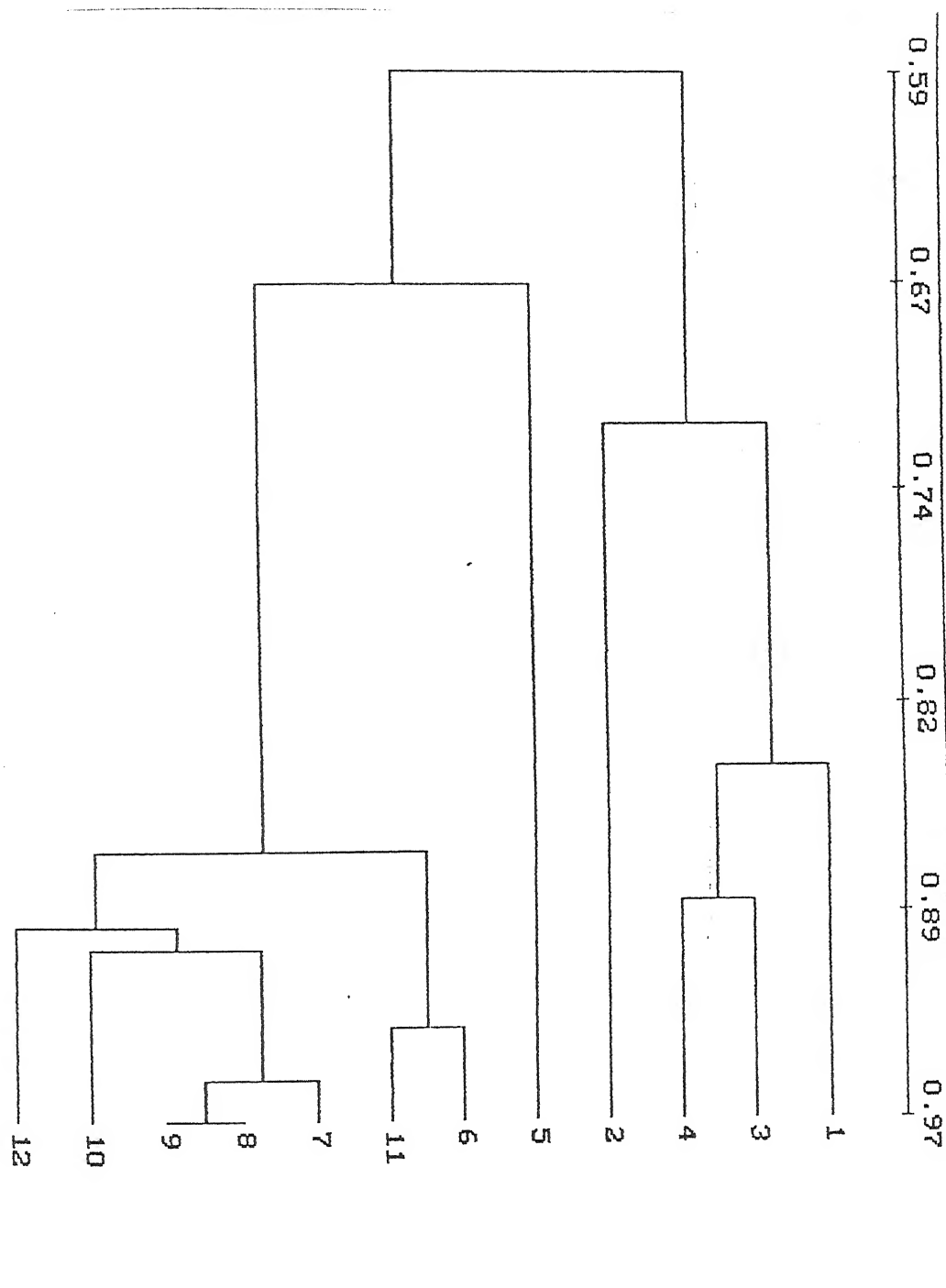
Fig 17. Clustering based on isozyme data showing genetic relatedness among accessions of *Trifolium resupinatum*.



List of accessions

- | | | | | | |
|-------------|-------------|-------------|--------------|--------------|--------------|
| 1. SH 98-36 | 2. SH 98-72 | 3. SH 98-73 | 4. SH 98-86 | 5. JHS -3 | 6. SH 99-29 |
| 7. SH 99-69 | 8. SH 99-23 | 9. SH 99-33 | 10. SH 99-32 | 11. SH 99-25 | 12. SH 99-26 |

Fig 18 : Dendrogram based on cluster analysis of isozyme data showing genetic relatedness between *T. resupinatum* accessions.



T. resupinatum accessions

- | | | | | | |
|-------------|-------------|-------------|--------------|--------------|--------------|
| 1. SH 98-36 | 2. SH 98-72 | 3. SH 98-73 | 4. SH 98-86 | 5. JHS -3 | 6. SH 99-29 |
| 7. SH 99-69 | 8. SH 99-23 | 9. SH 99-33 | 10. SH 99-32 | 11. SH 99-25 | 12. SH 99-26 |

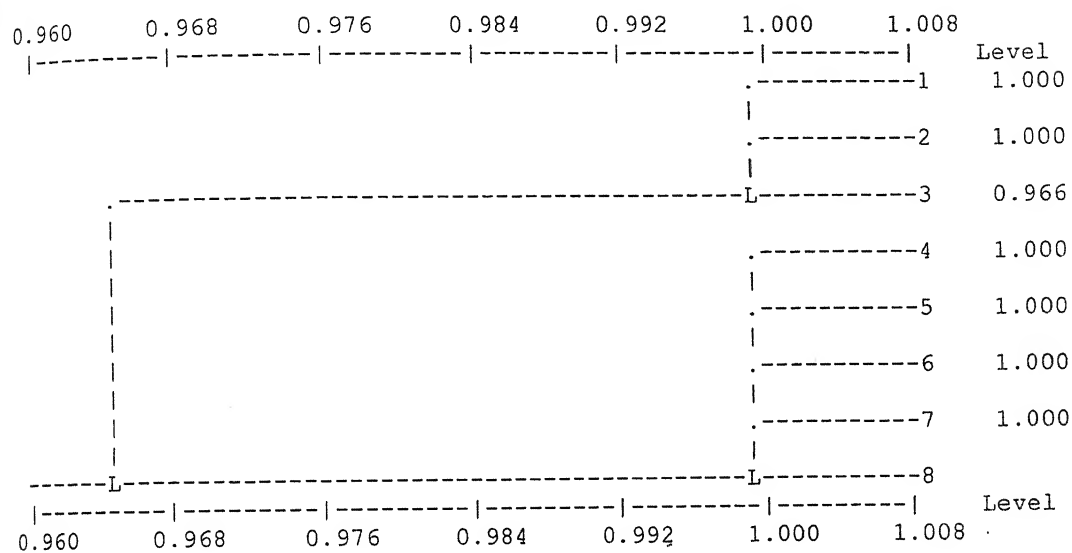
Table 4.31. Similarity matrix based on isozyme banding pattern in *Trifolium resupinatum* accessions

Sr. No.	Accession no.	1	2	3	4	5	6	7	8	9	10	11	12
1	SH 98-36	1.00											
2	SH 98-72	0.77	1.00										
3	SH 98-73	0.87	0.69	1.00									
4	SH 98-86	0.82	0.70	0.89	1.00								
5	JHS -3	0.70	0.67	0.70	0.59	1.00							
6	SH 99-29	0.53	0.54	0.60	0.67	0.65	1.00						
7	SH 99-69	0.58	0.52	0.65	0.64	0.68	0.90	1.00					
8	SH 99-23	0.58	0.52	0.58	0.64	0.68	0.90	0.94	1.00				
9	SH 99-33	0.53	0.54	0.60	0.67	0.65	0.93	0.97	0.97	1.00			
10	SH 99-32	0.65	0.59	0.58	0.57	0.68	0.84	0.94	0.88	0.90	1.00		
11	SH 99-25	0.53	0.62	0.53	0.59	0.65	0.93	0.84	0.84	0.87	0.84	1.00	
12	SH 99-26	0.58	0.59	0.52	0.57	0.68	0.84	0.88	0.94	0.90	0.88	0.90	1.00

Table 4.32. Similarity matrix based on isozyme banding pattern in *Trifolium pratense* accessions

	Accession no.	1	2	3	4	5	6	7	8
1	EC 400979	1.00							
2	PRC -3	1.00	1.00						
3	EC 400980	1.00	1.00	1.00					
4	EC 400982	0.97	0.97	0.97	1.00				
5	EC 401721	0.97	0.97	0.97	1.00	1.00			
6	EC 401719	0.97	0.97	0.97	1.00	1.00	1.00		
7	EC 401720	0.97	0.97	0.97	1.00	1.00	1.00	1.00	
8	EC 400735	0.97	0.97	0.97	1.00	1.00	1.00	1.00	1.00

Fig. 19. Clustering based on isozyme data showing relatedness among *Trifolium pratense* accessions



List of accessions

- | | | | |
|--------------|--------------|--------------|--------------|
| 1. EC 400979 | 2. PRC - 3 | 3. EC 400980 | 4. EC 400982 |
| 5. EC 401721 | 6. EC 401719 | 7. EC 401720 | 8. EC 400735 |

Cluster	Accessions
1	EC 400979, PRC-3, EC 400980
2	EC 400982, EC 401721, EC 401719, EC 401720, EC 400735.

T. alexandrinum

Cluster analysis was done on the basis of five enzyme system of 65 germplasm lines of *T. alexandrinum*. Total 13 clusters were observed (Table 4.34; Fig.20,21). Cluster number 1 comprising of single accession *i.e.* EC 329299 showed 79% similarity with rest of the clusters. All remaining clusters showed more than 90% similarity among themselves. Cluster number 2 comprised of seven lines, out of which six lines showed 100% similarity and together they were 97.7% similar with IL 40010.

A total of 13 lines were observed in cluster number 3, out of which 10 lines were observed for 100% similarity in first group and remaining 3 lines were also 100% similar in the second group of the same cluster. The two groups showed 97% similarity. Cluster number 3 showed 96.6% similarity cluster no. 2.

In cluster 4, JB 92-1 showed 98% similarity with two accessions *i.e.* EC 400977 and Raj 7/53-54-2 that were 100% similar. This cluster showed 95% similarity with combination of cluster no. 2 and 3. In cluster number 5, two similar lines showed 97% similarity with other 5 lines which were 100 % similar. This cluster was observed to be 95.2% similar with group of cluster no. 2,3 and 4.

Cluster number 6 showed 95% similarity between two lines and was 93% similar with group of cluster no. 7, 8 and 9. In cluster number 7, BL 122 was 100% similar to Raj 7/49-50 and these two accessions showed 98% similarity with JHB 146. In cluster number 8, five lines, which were 100% similar, showed 97% similarity with group of two lines which were also 100% similar (Table 4.33). This cluster showed 96.6% similarity with cluster no. 9.

In cluster number 9, eight lines were found which showed 100% similarity. This cluster showed 96.6% similarity with cluster number 8. The group comprising of cluster no. 2 to 9 showed 89.8 % similarity with another group of cluster no. 10 to 13. Cluster number 10, in which 4 lines were included in to two sub groups of

Table 4.33. Similarity matrix based on isozyme banding pattern in *T. alexandrinum* genotypes

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1.00															
2	0.86	1.00														
3	0.85	0.95	1.00						List of genotypes is given at end of the table							
4	0.85	0.95	1.00	1.00												
5	0.85	0.95	1.00	1.00	1.00											
6	0.85	0.95	1.00	1.00	1.00	1.00										
7	0.85	0.95	1.00	1.00	1.00	1.00	1.00									
8	0.81	0.96	0.95	0.95	0.95	0.95	0.95	1.00								
9	0.85	0.95	1.00	1.00	1.00	1.00	1.00	0.95	1.00							
10	0.83	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	1.00						
11	0.75	0.86	0.90	0.90	0.90	0.90	0.90	0.91	0.90	0.88	1.00					
12	0.74	0.85	0.90	0.90	0.90	0.90	0.90	0.85	0.90	0.87	0.95	1.00				
13	0.70	0.82	0.87	0.87	0.87	0.87	0.87	0.82	0.87	0.84	0.92	0.97	1.00			
14	0.68	0.80	0.84	0.84	0.84	0.84	0.84	0.85	0.84	0.82	0.95	0.94	0.97	1.00		
15	0.74	0.85	0.90	0.90	0.90	0.90	0.90	0.85	0.90	0.87	0.95	1.00	0.97	0.94	1.00	
16	0.72	0.83	0.87	0.87	0.87	0.87	0.87	0.88	0.87	0.85	0.97	0.97	0.94	0.97	0.97	1.00
17	0.77	0.83	0.87	0.87	0.87	0.87	0.87	0.88	0.87	0.85	0.92	0.92	0.89	0.92	0.92	0.95
18	0.74	0.85	0.90	0.90	0.90	0.90	0.90	0.85	0.90	0.87	0.95	1.00	0.97	0.94	1.00	0.97
19	0.77	0.88	0.92	0.92	0.92	0.92	0.92	0.88	0.92	0.90	0.97	0.97	0.94	0.92	0.97	0.95
20	0.72	0.83	0.87	0.87	0.87	0.87	0.87	0.88	0.87	0.85	0.97	0.97	0.94	0.97	0.97	1.00
21	0.70	0.82	0.87	0.87	0.87	0.87	0.87	0.82	0.87	0.84	0.92	0.97	1.00	0.97	0.97	0.94
22	0.70	0.82	0.87	0.87	0.87	0.87	0.87	0.82	0.87	0.84	0.92	0.97	1.00	0.97	0.97	0.94
23	0.83	0.93	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.95	0.93	0.87	0.84	0.87	0.87	0.90
24	0.78	0.88	0.93	0.93	0.93	0.93	0.93	0.93	0.93	0.91	0.93	0.92	0.90	0.92	0.92	0.95
25	0.80	0.91	0.95	0.95	0.95	0.95	0.95	0.91	0.95	0.93	0.90	0.95	0.92	0.90	0.95	0.92
26	0.78	0.88	0.93	0.93	0.93	0.93	0.93	0.93	0.93	0.91	0.93	0.92	0.90	0.92	0.92	0.95
27	0.83	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	1.00	0.88	0.87	0.84	0.82	0.87	0.85
28	0.85	0.95	1.00	1.00	1.00	1.00	1.00	0.95	1.00	0.98	0.90	0.90	0.87	0.84	0.90	0.87
29	0.83	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	1.00	0.88	0.87	0.84	0.82	0.87	0.85
30	0.85	0.95	1.00	1.00	1.00	1.00	1.00	0.95	1.00	0.98	0.90	0.90	0.87	0.84	0.90	0.87
31	0.83	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	1.00	0.88	0.87	0.84	0.82	0.87	0.85
32	0.85	0.95	1.00	1.00	1.00	1.00	1.00	0.95	1.00	0.98	0.90	0.90	0.87	0.84	0.90	0.87
33	0.85	0.95	1.00	1.00	1.00	1.00	1.00	0.95	1.00	0.98	0.90	0.90	0.87	0.84	0.90	0.87
34	0.83	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	1.00	0.88	0.87	0.84	0.82	0.87	0.85
35	0.83	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	1.00	0.88	0.87	0.84	0.82	0.87	0.85
36	0.81	0.96	0.95	0.95	0.95	0.95	0.95	1.00	0.95	0.98	0.91	0.85	0.82	0.85	0.85	0.88
37	0.75	0.86	0.90	0.90	0.90	0.90	0.90	0.91	0.90	0.88	0.95	0.95	0.92	0.95	0.95	0.97
38	0.77	0.88	0.92	0.92	0.92	0.92	0.92	0.93	0.92	0.90	0.92	0.92	0.89	0.92	0.92	0.95
39	0.78	0.93	0.93	0.93	0.93	0.93	0.93	0.93	0.93	0.95	0.93	0.92	0.90	0.87	0.92	0.90
40	0.77	0.88	0.92	0.92	0.92	0.92	0.92	0.93	0.92	0.90	0.92	0.92	0.89	0.92	0.92	0.95
41	0.78	0.88	0.93	0.93	0.93	0.93	0.93	0.93	0.93	0.91	0.98	0.92	0.90	0.92	0.92	0.95
42	0.79	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92
43	0.80	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.98	0.85	0.90	0.87	0.84	0.90	0.87
44	0.80	0.91	0.95	0.95	0.95	0.95	0.95	0.91	0.95	0.93	0.95	0.95	0.92	0.90	0.95	0.92
45	0.75	0.86	0.90	0.90	0.90	0.90	0.90	0.91	0.90	0.88	0.95	0.95	0.92	0.95	0.95	0.97
46	0.77	0.93	0.92	0.92	0.92	0.92	0.92	0.93	0.92	0.95	0.87	0.92	0.89	0.87	0.92	0.90
47	0.79	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92
48	0.77	0.93	0.92	0.92	0.92	0.92	0.92	0.93	0.92	0.95	0.87	0.92	0.89	0.87	0.92	0.90
49	0.77	0.88	0.92	0.92	0.92	0.92	0.92	0.93	0.92	0.90	0.92	0.92	0.89	0.92	0.92	0.95
50	0.77	0.88	0.92	0.92	0.92	0.92	0.92	0.93	0.92	0.90	0.92	0.92	0.89	0.92	0.92	0.95
51	0.77	0.88	0.92	0.92	0.92	0.92	0.92	0.93	0.92	0.90	0.92	0.92	0.89	0.92	0.92	0.95
52	0.79	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92
53	0.79	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92
54	0.77	0.88	0.87	0.87	0.87	0.87	0.87	0.93	0.87	0.90	0.87	0.87	0.83	0.87	0.87	0.90
55	0.81	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92
56	0.82	0.93	0.97	0.97	0.97	0.97	0.97	0.93	0.97	0.95	0.87	0.92	0.89	0.87	0.92	0.90
57	0.82	0.93	0.97	0.97	0.97	0.97	0.97	0.93	0.97	0.95	0.87	0.92	0.89	0.87	0.92	0.90
58	0.82	0.93	0.97	0.97	0.97	0.97	0.97	0.93	0.97	0.95	0.87	0.92	0.89	0.87	0.92	0.90
59	0.79	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92
60	0.79	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92
61	0.79	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92
62	0.80	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.98	0.85	0.90	0.87	0.84	0.90	0.87
63	0.80	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.98	0.85	0.90	0.87	0.84	0.90	0.87
64	0.80	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.98	0.85	0.90	0.87	0.84	0.90	0.87
65	0.80	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.98	0.85	0.90	0.87	0.84	0.90	0.87

Table 4.33. (Contd.) Similarity matrix based on isozyme banding pattern in *T. alexandrinum* genotypes

	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1																
2																
3																
4																
5																
6																
7																
8																
9																
10																
11																
12																
13																
14																
15																
16																
17	1.00															
18	0.92	1.00														
19	0.90	0.97	1.00													
20	0.95	0.97	0.95	1.00												
21	0.89	0.97	0.94	0.94	1.00											
22	0.89	0.97	0.94	0.94	1.00	1.00										
23	0.90	0.87	0.90	0.90	0.84	0.84	1.00									
24	0.95	0.92	0.90	0.95	0.90	0.90	0.95	1.00								
25	0.92	0.95	0.92	0.92	0.92	0.92	0.93	0.98	1.00							
26	0.95	0.92	0.90	0.95	0.90	0.90	0.95	1.00	0.98	1.00						
27	0.85	0.87	0.90	0.85	0.84	0.84	0.95	0.91	0.93	0.91	1.00					
28	0.87	0.90	0.92	0.87	0.87	0.87	0.98	0.93	0.95	0.93	0.98	1.00				
29	0.85	0.87	0.90	0.85	0.84	0.84	0.95	0.91	0.93	0.91	1.00	0.98	1.00			
30	0.87	0.90	0.92	0.87	0.87	0.87	0.98	0.93	0.95	0.93	0.98	1.00	0.98	1.00		
31	0.85	0.87	0.90	0.85	0.84	0.84	0.95	0.91	0.93	0.91	1.00	0.98	1.00	0.98	1.00	
32	0.87	0.90	0.92	0.87	0.87	0.87	0.98	0.93	0.95	0.93	0.98	1.00	0.98	1.00	0.98	1.00
33	0.87	0.90	0.92	0.87	0.87	0.87	0.98	0.93	0.95	0.93	0.98	1.00	0.98	1.00	0.98	1.00
34	0.85	0.87	0.90	0.85	0.84	0.84	0.95	0.91	0.93	0.91	1.00	0.98	1.00	0.98	1.00	0.98
35	0.85	0.87	0.90	0.85	0.84	0.84	0.95	0.91	0.93	0.91	1.00	0.98	1.00	0.98	1.00	0.98
36	0.88	0.85	0.88	0.88	0.82	0.82	0.98	0.93	0.91	0.93	0.98	0.95	0.98	0.95	0.98	0.95
37	0.97	0.95	0.92	0.97	0.92	0.92	0.93	0.98	0.95	0.98	0.88	0.90	0.88	0.90	0.88	0.90
38	0.95	0.92	0.90	0.95	0.89	0.89	0.95	0.95	0.92	0.95	0.90	0.92	0.90	0.92	0.90	0.92
39	0.90	0.92	0.95	0.90	0.90	0.90	0.91	0.91	0.93	0.91	0.95	0.93	0.95	0.93	0.95	0.93
40	0.95	0.92	0.90	0.95	0.89	0.89	0.95	0.95	0.92	0.95	0.90	0.92	0.90	0.92	0.90	0.92
41	0.95	0.92	0.95	0.95	0.90	0.90	0.95	0.95	0.93	0.95	0.91	0.93	0.91	0.93	0.91	0.93
42	0.92	0.94	0.92	0.92	0.91	0.91	0.92	0.92	0.95	0.92	0.92	0.95	0.92	0.95	0.92	0.95
43	0.87	0.90	0.87	0.87	0.87	0.87	0.93	0.93	0.95	0.93	0.98	0.95	0.98	0.95	0.98	0.95
44	0.92	0.95	0.97	0.92	0.92	0.92	0.93	0.93	0.95	0.93	0.93	0.95	0.93	0.95	0.93	0.95
45	0.97	0.95	0.92	0.97	0.92	0.92	0.93	0.98	0.95	0.98	0.88	0.90	0.88	0.90	0.88	0.90
46	0.90	0.92	0.90	0.90	0.89	0.89	0.90	0.90	0.92	0.90	0.95	0.92	0.95	0.92	0.95	0.92
47	0.92	0.94	0.92	0.92	0.91	0.91	0.92	0.92	0.95	0.92	0.92	0.95	0.92	0.95	0.92	0.95
48	0.90	0.92	0.90	0.90	0.89	0.89	0.90	0.90	0.92	0.90	0.95	0.92	0.95	0.92	0.95	0.92
49	0.95	0.92	0.90	0.95	0.89	0.89	0.95	0.95	0.92	0.95	0.90	0.92	0.90	0.92	0.90	0.92
50	0.95	0.92	0.90	0.95	0.89	0.89	0.95	0.95	0.92	0.95	0.90	0.92	0.90	0.92	0.90	0.92
51	0.95	0.92	0.90	0.95	0.89	0.89	0.95	0.95	0.92	0.95	0.90	0.92	0.90	0.92	0.90	0.92
52	0.92	0.94	0.92	0.92	0.91	0.91	0.92	0.92	0.95	0.92	0.92	0.95	0.92	0.95	0.92	0.95
53	0.92	0.94	0.92	0.92	0.91	0.91	0.92	0.92	0.95	0.92	0.92	0.95	0.92	0.95	0.92	0.95
54	0.95	0.87	0.84	0.90	0.83	0.83	0.90	0.90	0.87	0.90	0.90	0.87	0.90	0.87	0.90	0.87
55	0.92	0.94	0.92	0.92	0.91	0.91	0.92	0.92	0.95	0.92	0.92	0.95	0.92	0.95	0.92	0.95
56	0.90	0.92	0.90	0.90	0.89	0.89	0.95	0.95	0.97	0.95	0.95	0.97	0.95	0.97	0.95	0.97
57	0.90	0.92	0.90	0.90	0.89	0.89	0.95	0.95	0.97	0.95	0.95	0.97	0.95	0.97	0.95	0.97
58	0.90	0.92	0.90	0.90	0.89	0.89	0.95	0.95	0.97	0.95	0.95	0.97	0.95	0.97	0.95	0.97
59	0.92	0.94	0.92	0.92	0.91	0.91	0.92	0.92	0.95	0.92	0.92	0.95	0.92	0.95	0.92	0.95
60	0.92	0.94	0.92	0.92	0.91	0.91	0.92	0.92	0.95	0.92	0.92	0.95	0.92	0.95	0.92	0.95
61	0.92	0.94	0.92	0.92	0.91	0.91	0.92	0.92	0.95	0.92	0.92	0.95	0.92	0.95	0.92	0.95
62	0.87	0.90	0.87	0.87	0.87	0.87	0.93	0.93	0.95	0.93	0.98	0.95	0.98	0.95	0.98	0.95
63	0.87	0.90	0.87	0.87	0.87	0.87	0.93	0.93	0.95	0.93	0.98	0.95	0.98	0.95	0.98	0.95
64	0.87	0.90	0.87	0.87	0.87	0.87	0.93	0.93	0.95	0.93	0.98	0.95	0.98	0.95	0.98	0.95
65	0.87	0.90	0.87	0.87	0.87	0.87	0.93	0.93	0.95	0.93	0.98	0.95	0.98	0.95	0.98	0.95

Table 4.33. (Contd..) Similarity matrix based on isozyme banding pattern in *T. alexandrinum* genotypes

	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1																
2																
3																
4																
5																
6																
7																
8																
9																
10																
11																
12																
13																
14																
15																
16																
17																
18																
19																
20																
21																
22																
23																
24																
25																
26																
27																
28																
29																
30																
31																
32																
33	1.00															
34	0.98	1.00														
35	0.98	1.00	1.00													
36	0.95	0.98	0.98	1.00												
37	0.90	0.88	0.88	0.91	1.00											
38	0.92	0.90	0.90	0.93	0.97	1.00										
39	0.93	0.95	0.95	0.93	0.93	0.90	1.00									
40	0.92	0.90	0.90	0.93	0.97	1.00	0.90	1.00								
41	0.93	0.91	0.91	0.93	0.98	0.95	0.95	0.95	1.00							
42	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00						
43	0.95	0.98	0.98	0.95	0.90	0.92	0.93	0.92	0.88	0.95	1.00					
44	0.95	0.93	0.93	0.91	0.95	0.92	0.98	0.92	0.98	0.95	0.90	1.00				
45	0.90	0.88	0.88	0.91	1.00	0.97	0.93	0.97	0.98	0.95	0.90	0.95	1.00			
46	0.92	0.95	0.95	0.93	0.92	0.95	0.95	0.95	0.90	0.97	0.97	0.92	0.92	1.00		
47	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00	0.95	0.95	0.95	0.97	1.00	
48	0.92	0.95	0.95	0.93	0.92	0.95	0.95	0.95	0.90	0.97	0.97	0.92	0.92	1.00	0.97	1.00
49	0.92	0.90	0.90	0.93	0.97	1.00	0.90	1.00	0.95	0.97	0.92	0.92	0.97	0.95	0.97	0.95
50	0.92	0.90	0.90	0.93	0.97	1.00	0.90	1.00	0.95	0.97	0.92	0.92	0.97	0.95	0.97	0.95
51	0.92	0.90	0.90	0.93	0.97	1.00	0.90	1.00	0.95	0.97	0.92	0.92	0.97	0.95	0.97	0.95
52	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00	0.95	0.95	0.95	0.97	1.00	0.97
53	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00	0.95	0.95	0.95	0.97	1.00	0.97
54	0.87	0.90	0.90	0.93	0.92	0.95	0.90	0.95	0.90	0.92	0.92	0.87	0.92	0.95	0.92	0.95
55	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00	0.95	0.95	0.95	0.97	1.00	0.97
56	0.97	0.95	0.95	0.93	0.92	0.95	0.90	0.95	0.90	0.97	0.97	0.92	0.92	0.95	0.97	0.95
57	0.97	0.95	0.95	0.93	0.92	0.95	0.90	0.95	0.90	0.97	0.97	0.92	0.92	0.95	0.97	0.95
58	0.97	0.95	0.95	0.93	0.92	0.95	0.90	0.95	0.90	0.97	0.97	0.92	0.92	0.95	0.97	0.95
59	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00	0.95	0.95	0.95	0.97	1.00	0.97
60	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00	0.95	0.95	0.95	0.97	1.00	0.97
61	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00	0.95	0.95	0.95	0.97	1.00	0.97
62	0.95	0.98	0.98	0.95	0.90	0.92	0.93	0.92	0.88	0.95	1.00	0.90	0.90	0.97	0.95	0.97
63	0.95	0.98	0.98	0.95	0.90	0.92	0.93	0.92	0.88	0.95	1.00	0.90	0.90	0.97	0.95	0.97
64	0.95	0.98	0.98	0.95	0.90	0.92	0.93	0.92	0.88	0.95	1.00	0.90	0.90	0.97	0.95	0.97
65	0.95	0.98	0.98	0.95	0.90	0.92	0.93	0.92	0.88	0.95	1.00	0.90	0.90	0.97	0.95	0.97

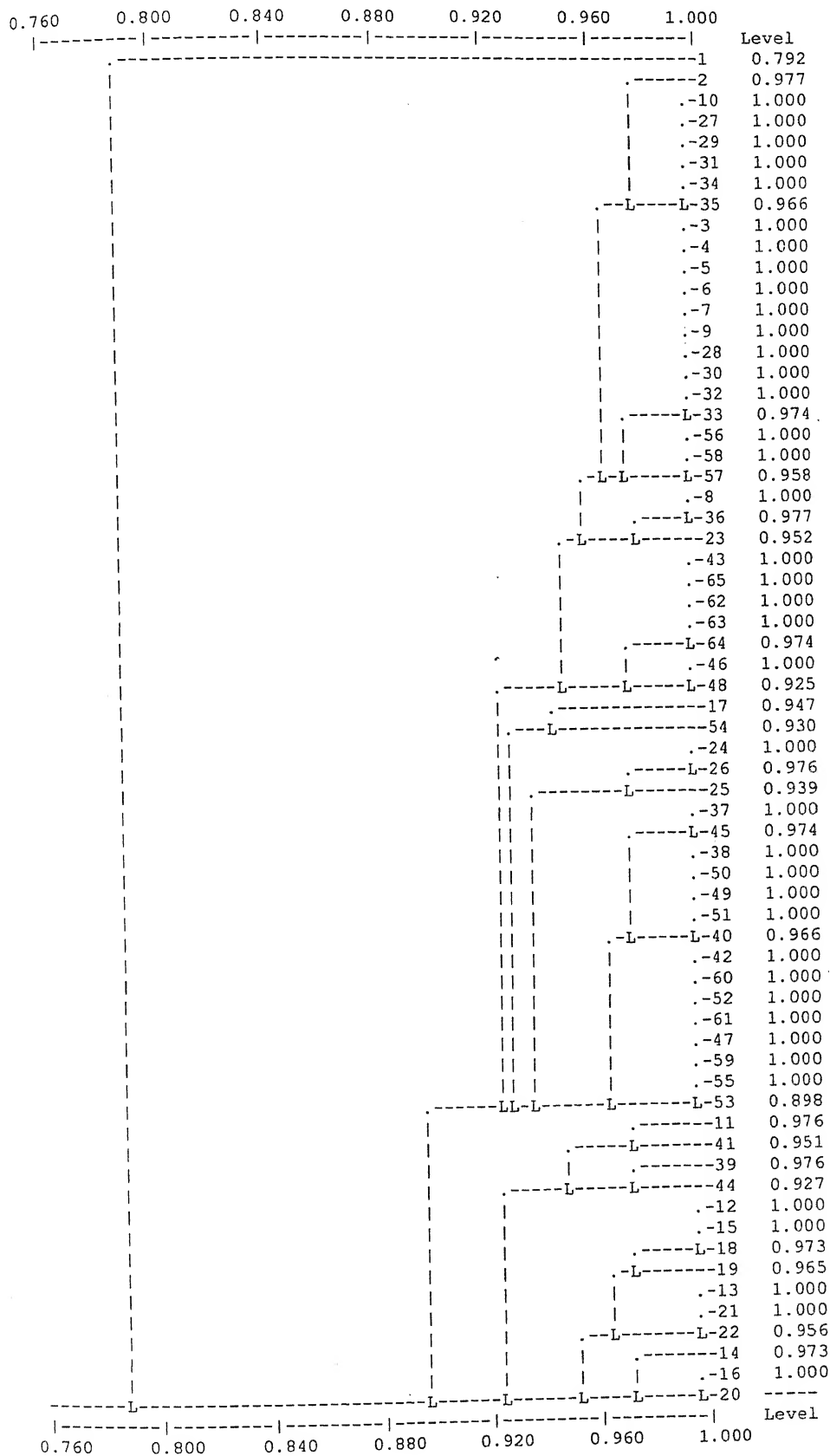
Table 4.33. (Contd..) Similarity matrix based on isozyme banding pattern in *T. alexandrinum* genotypes

	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
1																	
2																	
3																	
4																	
5																	
			S.N.			Genotypes				S.N.			Genotypes				
6			1			EC 329299				34			JHTB-1-90-P3				
7			2			IL 40010				35			Raj 7/53-54 - O				
8			3			EC 400733				36			Raj 7/53-54 -2				
9			4			EC 401710				37			Raj 7/13-14 - O				
10			5			EC 401709				38			JHTB 5-90-I				
11			6			Wardan				39			IL 40010-Mes				
12			7			EC 402161				40			JHB 94 -18/11				
13			8			EC 400977				41			JHB 91 P-20				
14			9			EC 400976				42			JHB 34/22				
15			10			EC 401711				43			Raj - Bundi - O				
16			11			JHB 94 P-22				44			JHB -P- 23/35				
17			12			JHB 94 -R-16				45			JHTB -1-90-A1				
18			13			JHB 94 -R-35				46			JHB 94 -31				
19			14			JHB 94 -R-13				47			JHB 94 -25				
20			15			JHB 94 -R-25				48			IL 40014				
21			16			JHB 94 P/T -34				49			IL 40013				
22			17			EC 318951				50			JHB 94-P-60				
23			18			JHB 57P3				51			BL 144				
24			19			JHB P17-1				52			JHB94-56				
25			20			Raj 7/13-14				53			BL 142				
26			21			JHB 15-27				54			Raj 7/13-25				
27			22			JHB6/54 p/t				55			HFB 155				
28			23			JB 92-1				56			BL 131				
29			24			BL 122				57			JHB 36/5-54				
30			25			JHB 146				58			JHB CT2 G/35				
31			26			Raj 7/49-50				59			JHB 6/54				
32			27			JHTB 9-90 N1				60			JHB 16/2				
33			28			JHB 5-13/12				61			HFB 155				
34			29			IL 4009				62			Wardan S-1				
35			30			JHTB 5-90-2				63			Wardan S-2				
36			31			JHTB 3-90-H				64			Wardan S-3				
37			32			JHTB 13-90-B				65			Wardan S-4				
38			33			Raj 7/53-54											
39																	
40																	
41																	
42																	
43																	
44																	
45																	
46																	
47																	
48																	
49	1.00																
50	1.00	1.00															
51	1.00	1.00	1.00														
52	0.97	0.97	0.97	1.00													
53	0.97	0.97	0.97	1.00	1.00												
54	0.95	0.95	0.95	0.92	0.92	1.00											
55	0.97	0.97	0.97	1.00	1.00	0.92	1.00										
56	0.95	0.95	0.95	0.97	0.97	0.90	0.97	1.00									
57	0.95	0.95	0.95	0.97	0.97	0.90	0.97	1.00	1.00								
58	0.95	0.95	0.95	0.97	0.97	0.90	0.97	1.00	1.00	1.00							
59	0.97	0.97	0.97	1.00	1.00	0.92	1.00	0.97	0.97	0.97	1.00						
60	0.97	0.97	0.97	1.00	1.00	0.92	1.00	0.97	0.97	0.97	1.00	1.00					
61	0.97	0.97	0.97	1.00	1.00	0.92	1.00	0.97	0.97	0.97	1.00	1.00	1.00				
62	0.92	0.92	0.92	0.95	0.95	0.92	0.95	0.97	0.97	0.97	0.95	0.95	0.95	1.00			
63	0.92	0.92	0.92	0.95	0.95	0.92	0.95	0.97	0.97	0.97	0.95	0.95	0.95	1.00	1.00		
64	0.92	0.92	0.92	0.95	0.95	0.92	0.95	0.97	0.97	0.97	0.95	0.95	0.95	1.00	1.00	1.00	
65	0.92	0.92	0.92	0.95	0.95	0.92	0.95	0.97	0.97	0.97	0.95	0.95	0.95	1.00	1.00	1.00	1.00

Table. 4.34. Clustering of 65 lines of *Trifolium alexandrinum* based on isozyme banding.

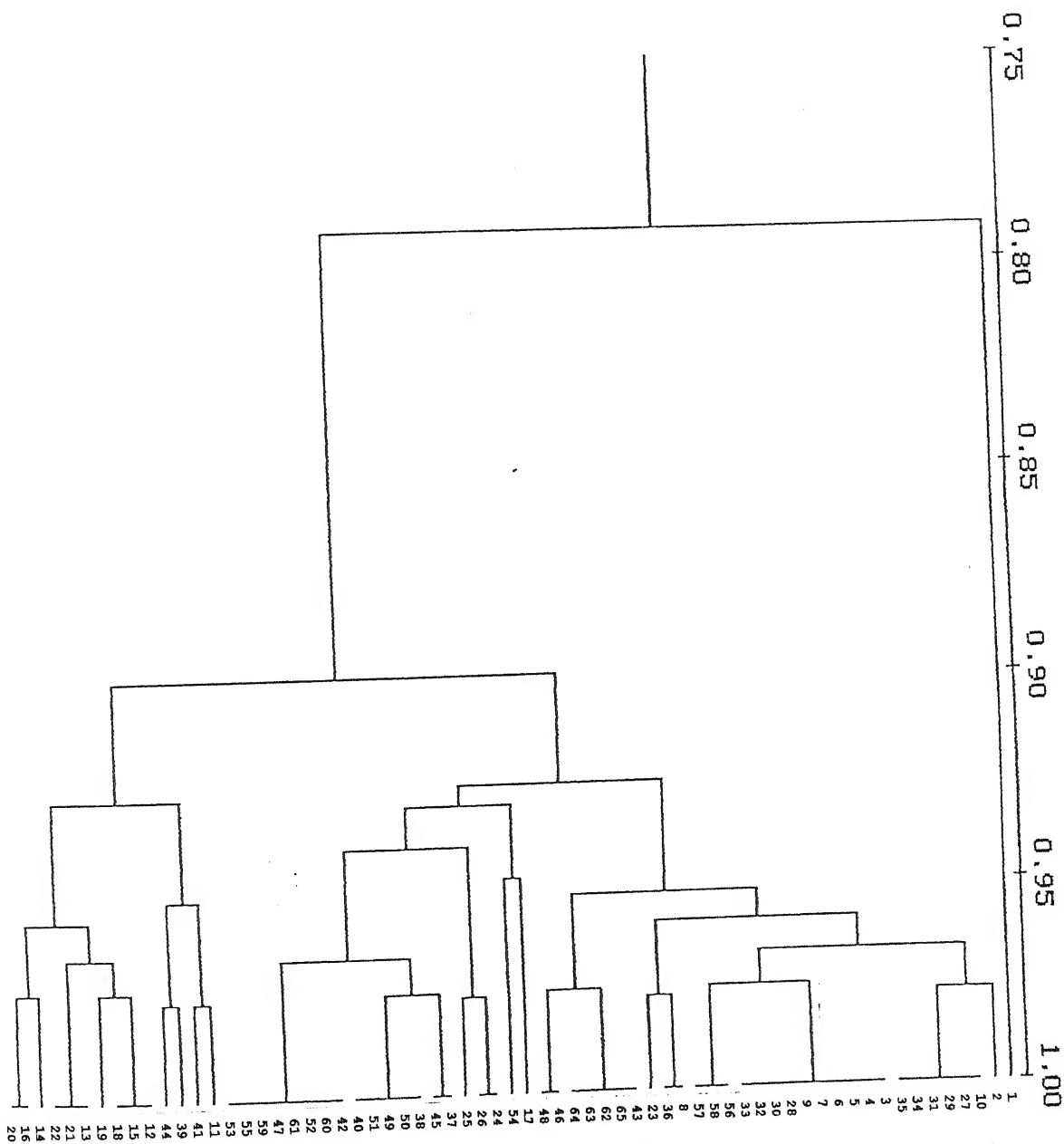
Cluster	Germplasm
1	EC 329299
2	IL 40010, EC 401711, JHTB 9-90N1, IL 4009, JHTB 3-90 H, JHTB -1-90-P3, Raj 7/53-54-O,
3	EC 400733, EC 401710, EC 401709, Warden, EC 402161, EC 400976, JHB 5-13/12, JHTB 5-90-2, JHTB-13-90-B, Raj 7/53-54, BL-131, JHB CT2 6/35, JHB36/5-54.
4	EC 400977, Raj 7/53-54-2, JB 92-1.
5	Raj-Bundi-O, Warden S-4, Warden S-1, Warden S-2, Warden S-3, JHB 94-31, IL 40014.
6	EC 318951, Raj 7/ 13-25.
7	BL 122, Raj 7/49-50, JHB 146.
8	Raj 7/13-14-O, JHTB-1-90-A1, JHTB 5-90I, JHB 94-P-60, IL 40013, BL 144, JHB 94-18/11.
9	JHB 34/22, JHB 16/2, JHB 94-56, HFB 155, JHB 94-25, JHB 6/54, HFB 155, BL 142.
10	JHB 94P-22, JHB 91P-20, IL 40010 Mes, JHB-P-23/35.
11	JHB 94-R-16, JHB94-R-25, JHB57P3, JHB P17-1.
12	JHB 94-R-35, JHB 15-27, JHB 6/54 p/t.
13	JHB 94-R-13, JHB 94P/T-34, Raj 7/13-14.

Fig. 20. Clustering based on isozyme data showing relatedness among *Trifolium alexandrinum* genotypes.



For details of accessions of *T. alexandrinum* (numbered from 1 to 65) refer to Fig.21.

Fig 21: Dendrogram based on cluster analysis of isozyme data showing genetic relatedness between *T. alexandrinum* accessions.



T. alexandrinum accessions

T. alexandrinum accessions
1.EC 329299, 2. IL 40010, 3.EC 400733, 4. EC 401710, 5. EC 401709, 6. Warden, 7. EC 402161, 8. EC 400977, 9. EC 400976, 10.EC 401711, 11. JHB 94P-22, 12. JHB94-R-16, 13. JHB94-R-35, 14. JHB94-R-13, 15. JHB94-R-25, 16. JHB94P-T-34, 17. EC 318951, 18. JHB 57P3, 19. JHB P17-1, 20. Raj 7/13-14, 21. JHB 15-27, 22. JHB 6/54 p/t, 23. JB92-JHB94P-T-34, 24. BL 122, 25. JHB 146, 26. Raj 7/49-50, 27. JHTB 9-90 N1, 28. JHB 5-13/12, 29. IL 4009, 30. JHTB 5-90-2, 31. JHTB 3-90-H, 32. JHTB 13-90-B, 33. Raj 7/ 53-54, 34. JHTB 1-90-P3, 35. Raj 7/53-54-O, 36. Raj 7/53-54-2, 37. Raj 7/13-14-O, 38. JHTB 5-90-I, 39. IL 40010-Mes.O, 40. JHB 94-18/I1, 41. JHB 91 P20, 42. JHB 34/22, 43. Raj-Bundi-O, 44. JHB -P-23/35, 45. JHTB 1-90-A1, 46. JHB 94-31, 47. JHB94-25, 48. IL 40014, 49. IL 40013, 50. JHB 94-P-60, 51. BL 144, 52. JHB 94-56, 53. BL 142, 54. Raj 7/13-25, 55. HFB 155, 56. BL 131, 57. JHB 36/5-54, 58. JHB CT2 6/35, 59. JHB 6/54, 60. JHB 16/2, 61. HFB 155, 62. Warden S-1, 63. Warden S-2, 64. Warden S-3, 65. Warden S-4,

Table 4.35. Similarity matrix based on isozyme pattern in different species of *Trifolium*

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1 <i>T. repens</i>	1.00																								
2 <i>T. pratense</i>	0.40	1.00																							
3 <i>T. subterraneum</i>	0.39	0.63	1.00																						
4 <i>T. nigrescens</i>	0.57	0.53	0.55	1.00																					
5 <i>T. hirtum</i>	0.47	0.46	0.53	0.49	1.00																				
6 <i>T. alpestre</i>	0.32	0.60	0.61	0.63	0.60	1.00																			
7 <i>T. echinatum</i>	0.59	0.55	0.51	0.57	0.55	0.58	1.00																		
8 <i>T. purpureum</i>	0.44	0.49	0.56	0.56	0.46	0.57	0.53	1.00																	
9 <i>T. campestre</i>	0.44	0.30	0.41	0.51	0.55	0.45	0.53	0.42	1.00																
10 <i>T. incarnatum</i>	0.47	0.41	0.49	0.39	0.44	0.49	0.45	0.36	0.55	1.00															
11 <i>T. chesteri</i>	0.50	0.69	0.58	0.59	0.51	0.53	0.55	0.54	0.42	0.51	1.00														
12 <i>T. lappaceum</i>	0.51	0.52	0.49	0.48	0.47	0.56	0.50	0.56	0.36	0.53	0.59	1.00													
13 <i>T. diffusum</i>	0.32	0.35	0.40	0.32	0.39	0.44	0.40	0.35	0.33	0.44	0.48	0.50	1.00												
14 <i>T. resupinatum</i>	0.51	0.56	0.57	0.58	0.48	0.49	0.59	0.58	0.41	0.52	0.61	0.47	0.30	1.00											
15 <i>T. angustifolium</i>	0.37	0.53	0.61	0.56	0.38	0.57	0.58	0.69	0.39	0.32	0.53	0.56	0.37	0.49	1.00										
16 <i>T. medium</i>	0.45	0.44	0.53	0.53	0.46	0.53	0.49	0.49	0.49	0.41	0.56	0.59	0.48	0.37	0.53	1.00									
17 <i>T. constantinopolitanum</i>	0.58	0.60	0.61	0.50	0.54	0.64	0.71	0.51	0.52	0.60	0.67	0.72	0.52	0.54	0.57	0.60	1.00								
18 <i>T. glomeratum</i>	0.55	0.50	0.42	0.59	0.56	0.53	0.49	0.43	0.49	0.51	0.63	0.52	0.41	0.56	0.40	0.63	0.60	1.00							
19 <i>T. alexandrinum</i>	0.58	0.55	0.68	0.65	0.63	0.62	0.63	0.69	0.50	0.59	0.64	0.52	0.46	0.72	0.53	0.51	0.58	0.60	1.00						
20 <i>T. retusum</i>	0.36	0.58	0.49	0.42	0.32	0.48	0.50	0.61	0.31	0.37	0.52	0.39	0.43	0.57	0.41	0.45	0.48	0.45	0.57	1.00					
21 <i>T. argutum</i>	0.40	0.38	0.37	0.53	0.46	0.53	0.49	0.43	0.49	0.51	0.44	0.30	0.28	0.61	0.40	0.31	0.40	0.56	0.64	0.45	1.00				
22 <i>T. spumosum</i>	0.34	0.61	0.56	0.57	0.50	0.65	0.53	0.47	0.47	0.55	0.79	0.43	0.47	0.50	0.45	0.55	0.58	0.49	0.63	0.63	0.42	1.00			
23 <i>T. temense</i>	0.44	0.49	0.56	0.46	0.55	0.52	0.53	0.53	0.53	0.45	0.61	0.57	0.47	0.50	0.58	0.55	0.71	0.49	0.58	0.44	0.49	0.53	1.00		
24 <i>T. apertum</i>	0.49	0.55	0.62	0.69	0.60	0.71	0.59	0.58	0.47	0.45	0.67	0.50	0.47	0.50	0.58	0.55	0.71	0.73	0.71	0.50	0.61	0.59	0.65	1.00	
25 <i>T. hybridum</i>	0.44	0.55	0.51	0.63	0.55	0.77	0.65	0.53	0.53	0.45	0.49	0.43	0.33	0.55	0.52	0.49	0.58	0.55	0.54	0.50	0.49	0.53	0.41	0.59	1.00

two lines each. The sub group of IL 40010-Mes and JHB P-23/35 showed 95% similarity with sub group of JHB 94P-22 and JHB 91P-20. This cluster showed 92.7% similarity with cluster 11, 12 and 13.

In cluster number 11, four lines were clustered and JHB P17-1 showed 97% similarity with group of 3 lines which were 100% similar. Cluster number 12 in which 3 lines were included, showed 100% intra cluster similarity. This cluster showed 96% similarity with cluster no. 11. In cluster number 13, three lines were included. JHB P/T-34 showed 100% similarity with Raj 7/13-14 and 97.3% similarity with JHB 94-R-13.

4.3.5.2 Interspecies similarity among different *Trifolium* species

Similarity among 25 species of *Trifolium* :

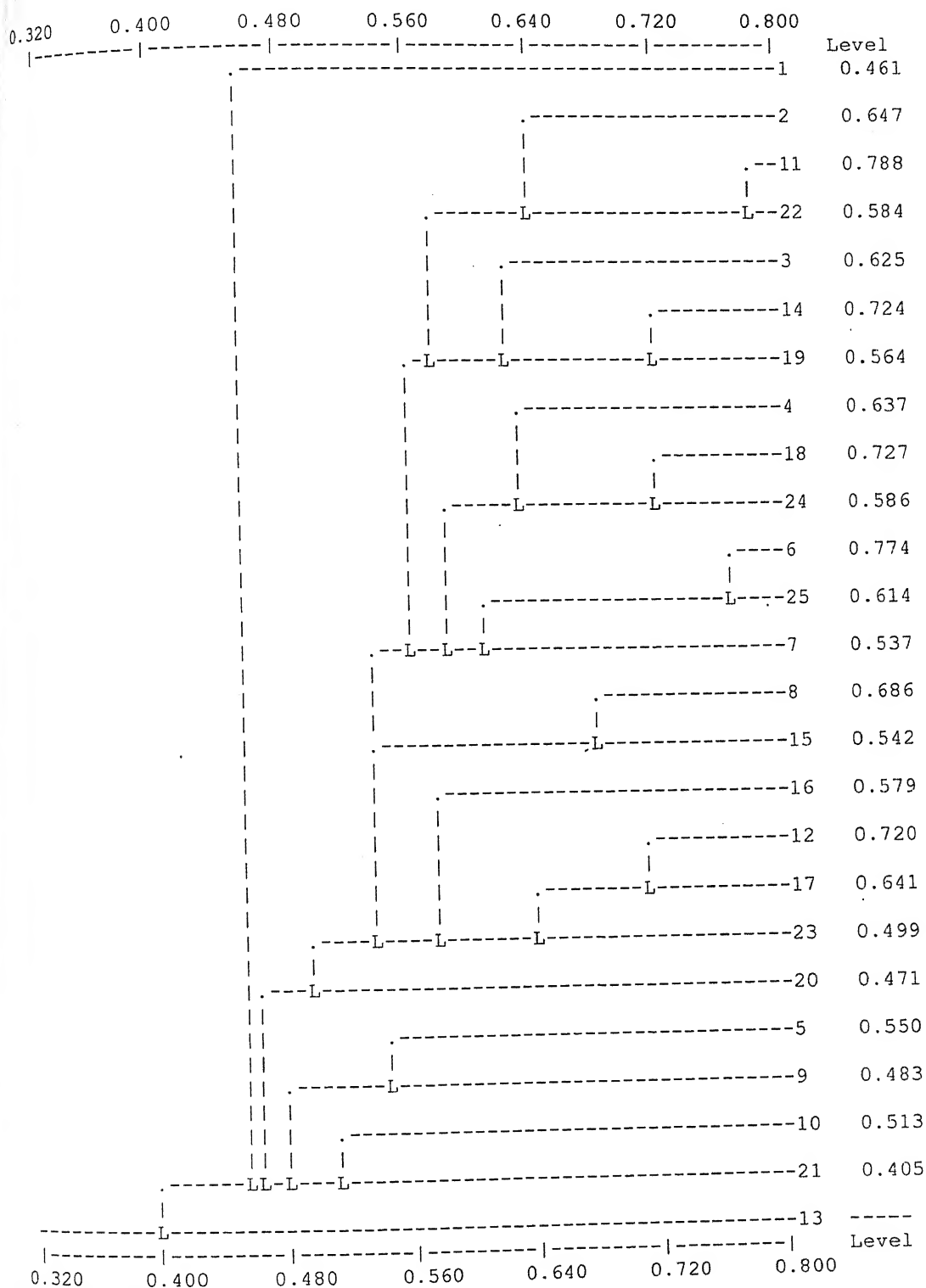
Dendrogram obtained on the basis of five enzyme system of 25 *Trifolium* species showed the presence of 7 clusters (Fig 22,23).

Table 4.36 : Clustering on basis of isozyme analysis in 25 species of *Trifolium*

Cluster no.	Species
1	<i>T. repens</i>
2	<i>T. pratense</i> , <i>T. cherleri</i> , <i>T. spumosum</i> , <i>T. subterraneum</i> , <i>T. resupinatum</i> , <i>T. alexandrinum</i>
3	<i>T. nigrescens</i> , <i>T. glomeratum</i> , <i>T. apertum</i> , <i>T. alpestre</i> , <i>T. hybridum</i> , <i>T. echinatum</i>
4	<i>T. purpureum</i> , <i>T. angustifolium</i> , <i>T. medium</i> , <i>T. lappaceum</i> , <i>T. constantinopolitanum</i> , <i>T. tembense</i>
5	<i>T. retusum</i>
6	<i>T. hirtum</i> , <i>T. campestre</i> , <i>T. incarnatum</i> , and <i>T. argutum</i> .
7	<i>T. diffusum</i> .

T. repens, *T. retusum* and *T. diffusum* each made clusters of single species i.e. clusters number 1, 5 and 7 respectively (Table 4.36). *T. repens* forming independent cluster showed 46% similarity with cluster no. 2 to 6. In cluster number 2, *T. spumosum* and *T. cherleri* showed highest affinity (78.8%) between the two species and these species together showed 64.7% similarity with *T. pratense*. In this cluster *T. alexandrinum* was 72.4% similar to *T. resupinatum* (Table 4.35). *T. subterraneum* was 62.5% similar with both *T. alexandrinum* and *T. resupinatum*.

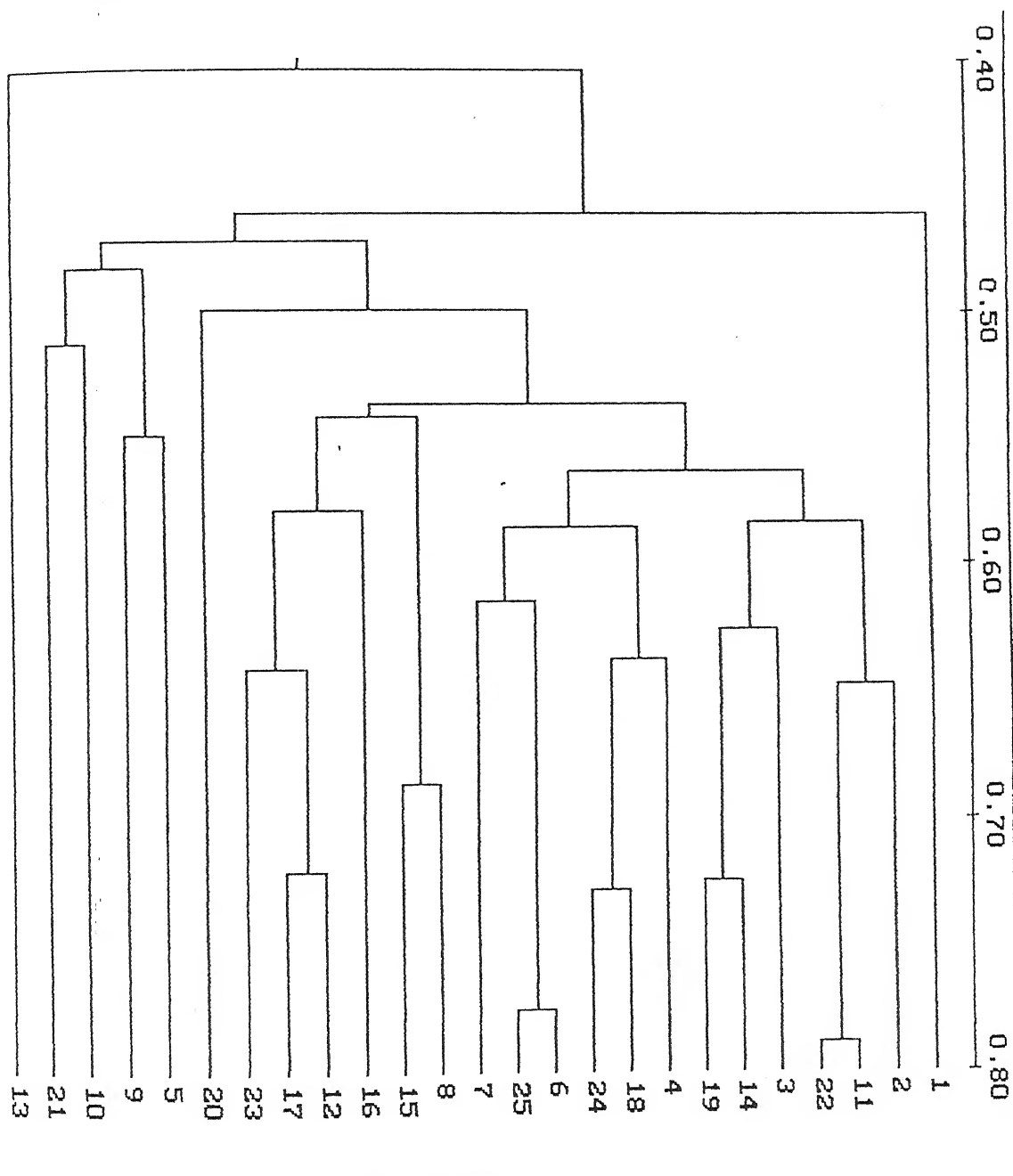
Fig.22. Clustering based on isozyme data showing genetic relatedness among 25 species of *Trifolium*.



List of species

1. *T. repens*, 2. *T. pratense*, 3. *T. subterraneum*, 4. *T. nigrescens*, 5. *T. hirtum*, 6. *T. alpestre*
7. *T. echinatum*, 8. *T. purpureum*, 9. *T. campestre*, 10. *T. incarnatum*, 11. *T. cherleri*,
12. *T. lappaceum*, 13. *T. diffusum*, 14. *T. resupinatum*, 15. *T. angustifolium*, 16. *T. medium*,
17. *T. constantinopolitanum*, 18. *T. glomeratum*, 19. *T. alexandrinum*, 20. *T. retusum*, 21. *T. argutum*,
22. *T. spumosum*, 23. *T. tembense*, 24. *T. apertum* 25. *T. hybridum*

Fig 23 : Dendrogram based on cluster analysis of isozyme data showing genetic relatedness among *Trifolium* species.



List of *Trifolium* species

1. *T. repens*, 2. *T. pratense*, 3. *T. subterraneum*, 4. *T. nigrescens*, 5. *T. hirtum*, 6. *T. alpestre*
 7. *T. echinatum*, 8. *T. purpureum*, 9. *T. campestre*, 10. *T. incarnatum*, 11. *T. cherleri*, 12. *T. lappaceum*,
 13. *T. diffusum*, 14. *T. resupinatum*, 15. *T. angustifolium*, 16. *T. medium*, 17. *T. constantinopolitanum*,
 18. *T. glomeratum*, 19. *T. alexandrinum*, 20. *T. retusum*, 21. *T. argutum*, 22. *T. spumosum*, 23. *T. lembense*,
 24. *T. apertum*, 25. *T. hybridum*

In cluster number 3, six species were clustered. Out of these *T. hybridum* and *T. alpestre* made a small subcluster of 77.4% similarity and 61.4% similarity with *T. echinatum*. Another sub-cluster of *T. apertum* and *T. glomeratum* and *T. nigrescens* was found which showed 63.7% similarity among themselves. The group of three species i.e. *T. hybridum*, *T. alpestre*, and *T. echinatum* showed 58.6% similarity with group of *T. apertum*, *T. glomeratum* and *T. nigrescens*. Cluster number 2 and 3 were observed to be 56.4 % similar.

In cluster 4, *T. angustifolium* showed 68.6% similarity with *T. purpureum* whereas *T. constantinopolitanum* was found 72% similar to *T. lappaceum*. 64 % similarity was observed between *T. tembense* and *T. constantinopolitanum*. 57.9% similarity was observed between *T. medium* and the other group of three species i.e. *T. constantinopolitanum*, *T. lappaceum* and *T. tembense*. Cluster no. 5 comprising of *T. retusum* showed 49.9% similarity with combination of cluster no. 2, 3 and 4.

Cluster number 6 comprised of 4 species and made two sub clusters. *T. campestre* was 55% similar to *T. hirtum* and *T. argutum* showed 51.3% similarity with *T. incarnatum*. These two sub clusters showed 48% similarity. Cluster no. 6 showed 47.1% similarity was group of clusters 2 to 5. Similarly, this group of clusters 2 to 6 was 46.1% similar with cluster no. 1. *T. diffusum* made separate cluster (No. 7) and showed only 40.5% similarity with rest of the clusters.

Similarity among 134 accessions of 25 *Trifolium* species :

The dendrogram obtained on the basis of five enzyme system of the 134 accessions of 25 *Trifolium* species showed the presence of 19 clusters (Table 4.38, Fig. 24,25).

Cluster no. 1 in which nine accessions of *T. repens* were included showed 48.9% similarity with group of cluster no. 2 to 16. In cluster no. 2, eight accessions of *T. pratense* were included and made two sub clusters. In one sub cluster, accessions i.e. EC 400979, PRC-3 and EC 400980 were 100% similar. Similarly, five accessions of other sub cluster i.e. EC 400982, EC 401721, EC 401719, EC 401720 and EC 400735 were also 100% similar (Table 4.37). 97% similarity was

Table 4.37. Similarity matrix based on isozyme pattern in 134 accessions of 25 <i>Trifolium</i> species																						
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
1	1.00																					
2	0.92	1.00																				
3	0.69	0.63	1.00																			
4	0.74	0.69	0.93	1.00																		
5	0.83	0.85	0.71	0.77	1.00																	
6	0.78	0.73	0.71	0.77	0.88	1.00																
7	0.78	0.73	0.71	0.77	0.88	1.00	1.00															
8	0.73	0.67	0.71	0.79	0.76	0.83	0.83	1.00														
9	0.73	0.67	0.71	0.79	0.76	0.83	0.83	1.00	1.00													
10	0.47	0.52	0.41	0.41	0.53	0.47	0.47	0.44	1.00	1.00												
11	0.47	0.52	0.41	0.41	0.53	0.47	0.47	0.44	1.00	1.00	1.00											
12	0.46	0.50	0.40	0.40	0.52	0.45	0.45	0.43	0.97	0.97	0.97	1.00										
13	0.46	0.50	0.40	0.40	0.52	0.45	0.45	0.43	0.97	0.97	0.97	1.00	1.00									
14	0.46	0.50	0.40	0.40	0.52	0.45	0.45	0.43	0.97	0.97	0.97	1.00	1.00	1.00								
15	0.46	0.50	0.40	0.40	0.52	0.45	0.45	0.43	0.97	0.97	0.97	1.00	1.00	1.00	1.00							
16	0.46	0.50	0.40	0.40	0.52	0.45	0.45	0.43	0.97	0.97	0.97	1.00	1.00	1.00	1.00	1.00						
17	0.46	0.50	0.40	0.40	0.52	0.45	0.45	0.43	0.97	0.97	0.97	1.00	1.00	1.00	1.00	1.00	1.00					
18	0.42	0.46	0.24	0.30	0.47	0.41	0.41	0.32	0.32	0.50	0.50	0.49	0.49	0.49	0.49	0.49	0.49	1.00				
19	0.42	0.46	0.24	0.30	0.47	0.41	0.41	0.32	0.32	0.50	0.50	0.49	0.49	0.49	0.49	0.49	0.49	1.00	1.00			
20	0.40	0.44	0.20	0.27	0.45	0.39	0.39	0.29	0.29	0.55	0.55	0.53	0.53	0.53	0.53	0.53	0.53	0.79	0.79	1.00		
21	0.43	0.47	0.31	0.38	0.49	0.42	0.42	0.40	0.40	0.58	0.58	0.56	0.56	0.56	0.56	0.56	0.56	0.91	0.91	0.75	1.00	
22	0.36	0.39	0.24	0.29	0.40	0.34	0.34	0.31	0.31	0.55	0.55	0.59	0.59	0.59	0.59	0.59	0.59	0.92	0.92	0.77	0.89	
23	0.50	0.55	0.44	0.44	0.57	0.43	0.43	0.40	0.40	0.39	0.39	0.37	0.37	0.37	0.37	0.37	0.37	0.47	0.47	0.52	0.41	
24	0.53	0.52	0.41	0.48	0.53	0.47	0.47	0.52	0.52	0.50	0.50	0.48	0.48	0.48	0.48	0.48	0.48	0.44	0.44	0.55	0.45	
25	0.52	0.57	0.46	0.46	0.59	0.44	0.44	0.42	0.42	0.40	0.40	0.39	0.39	0.39	0.39	0.39	0.39	0.41	0.41	0.46	0.36	
26	0.36	0.33	0.41	0.35	0.34	0.34	0.34	0.38	0.38	0.42	0.42	0.41	0.41	0.41	0.41	0.41	0.41	0.43	0.43	0.41	0.39	
27	0.39	0.36	0.45	0.39	0.38	0.38	0.38	0.41	0.41	0.47	0.47	0.45	0.45	0.45	0.45	0.45	0.45	0.41	0.41	0.45	0.36	
28	0.49	0.47	0.56	0.50	0.49	0.42	0.42	0.47	0.47	0.39	0.39	0.38	0.38	0.38	0.38	0.38	0.38	0.46	0.46	0.44	0.40	
29	0.38	0.41	0.30	0.30	0.43	0.36	0.36	0.32	0.32	0.54	0.54	0.52	0.52	0.52	0.52	0.52	0.53	0.53	0.52	0.48	0.52	
30	0.35	0.39	0.28	0.28	0.40	0.33	0.33	0.30	0.30	0.57	0.57	0.55	0.55	0.55	0.55	0.55	0.63	0.63	0.62	0.58	0.61	
31	0.61	0.67	0.45	0.52	0.63	0.63	0.63	0.55	0.55	0.53	0.53	0.52	0.52	0.52	0.52	0.52	0.47	0.47	0.52	0.49	0.46	
32	0.52	0.57	0.39	0.46	0.52	0.52	0.52	0.42	0.42	0.32	0.32	0.31	0.31	0.31	0.31	0.31	0.41	0.41	0.39	0.36	0.33	
33	0.45	0.43	0.34	0.40	0.50	0.44	0.44	0.49	0.49	0.41	0.41	0.40	0.40	0.40	0.40	0.40	0.53	0.53	0.51	0.54	0.46	
34	0.36	0.33	0.29	0.35	0.40	0.40	0.40	0.44	0.44	0.30	0.30	0.35	0.35	0.35	0.35	0.35	0.43	0.43	0.41	0.44	0.42	
35	0.29	0.31	0.40	0.40	0.32	0.26	0.26	0.29	0.29	0.28	0.28	0.27	0.27	0.27	0.27	0.27	0.42	0.42	0.40	0.44	0.41	
36	0.34	0.38	0.40	0.40	0.32	0.32	0.32	0.36	0.36	0.35	0.35	0.33	0.33	0.33	0.33	0.33	0.42	0.42	0.40	0.44	0.41	
37	0.48	0.51	0.38	0.43	0.42	0.32	0.32	0.34	0.34	0.44	0.44	0.43	0.43	0.43	0.43	0.43	0.55	0.55	0.43	0.56	0.54	
38	0.48	0.51	0.38	0.43	0.42	0.32	0.32	0.34	0.34	0.44	0.44	0.43	0.43	0.43	0.43	0.43	0.55	0.55	0.43	0.56	0.54	
39	0.56	0.55	0.39	0.45	0.50	0.44	0.44	0.48	0.48	0.36	0.36	0.35	0.35	0.35	0.35	0.35	0.71	0.71	0.47	0.65	0.51	
40	0.48	0.54	0.42	0.42	0.56	0.40	0.40	0.46	0.46	0.36	0.36	0.35	0.35	0.35	0.35	0.35	0.37	0.37	0.42	0.39	0.29	
41	0.31	0.35	0.22	0.22	0.36	0.29	0.29	0.16	0.16	0.39	0.39	0.37	0.37	0.37	0.37	0.37	0.47	0.47	0.52	0.41	0.39	
42	0.40	0.38	0.40	0.40	0.39	0.32	0.32	0.43	0.43	0.55	0.55	0.60	0.60	0.60	0.60	0.60	0.36	0.36	0.47	0.44	0.47	
43	0.39	0.36	0.39	0.39	0.37	0.30	0.30	0.42	0.42	0.56	0.56	0.54	0.54	0.54	0.54	0.54	0.41	0.41	0.54	0.43	0.47	
44	0.40	0.38	0.40	0.40	0.39	0.32	0.32	0.43	0.43	0.48	0.48	0.53	0.53	0.53	0.53	0.53	0.30	0.30	0.40	0.38	0.41	
45	0.38	0.35	0.37	0.37	0.36	0.29	0.29	0.40	0.40	0.39	0.39	0.44	0.44	0.44	0.44	0.44	0.33	0.33	0.37	0.35	0.39	

Similarity matrix for *Trifolium* accessions

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
46	0.52	0.51	0.43	0.49	0.53	0.42	0.42	0.46	0.46	0.56	0.56	0.56	0.60	0.60	0.60	0.60	0.60	0.50	0.49	0.56	0.59	
47	0.51	0.56	0.33	0.40	0.52	0.45	0.43	0.43	0.48	0.48	0.48	0.48	0.47	0.47	0.47	0.47	0.47	0.55	0.53	0.56	0.53	
48	0.56	0.61	0.45	0.52	0.63	0.56	0.56	0.55	0.55	0.53	0.53	0.53	0.52	0.52	0.52	0.52	0.52	0.53	0.53	0.52	0.61	
49	0.50	0.55	0.39	0.45	0.56	0.50	0.50	0.48	0.48	0.47	0.47	0.47	0.45	0.45	0.45	0.45	0.45	0.53	0.52	0.55	0.51	
50	0.51	0.56	0.40	0.47	0.58	0.52	0.52	0.50	0.50	0.48	0.48	0.48	0.47	0.47	0.47	0.47	0.47	0.55	0.53	0.56	0.53	
51	0.56	0.61	0.45	0.52	0.63	0.56	0.56	0.55	0.55	0.60	0.60	0.60	0.58	0.58	0.58	0.58	0.58	0.59	0.59	0.58	0.67	
52	0.51	0.56	0.33	0.40	0.52	0.45	0.43	0.43	0.48	0.48	0.48	0.47	0.47	0.47	0.47	0.47	0.47	0.55	0.53	0.56	0.57	
53	0.50	0.55	0.39	0.45	0.56	0.50	0.50	0.48	0.48	0.47	0.47	0.47	0.45	0.45	0.45	0.45	0.45	0.53	0.52	0.55	0.51	
54	0.41	0.45	0.28	0.35	0.47	0.40	0.40	0.37	0.37	0.43	0.43	0.43	0.48	0.48	0.48	0.48	0.48	0.50	0.50	0.62	0.52	
55	0.41	0.45	0.28	0.35	0.47	0.40	0.40	0.37	0.37	0.43	0.43	0.43	0.48	0.48	0.48	0.48	0.48	0.50	0.50	0.62	0.52	
56	0.44	0.49	0.32	0.38	0.50	0.38	0.38	0.28	0.28	0.47	0.47	0.47	0.45	0.45	0.45	0.45	0.45	0.53	0.53	0.58	0.42	
57	0.65	0.71	0.41	0.48	0.67	0.60	0.60	0.52	0.52	0.64	0.64	0.64	0.62	0.62	0.62	0.62	0.62	0.63	0.63	0.55	0.65	
58	0.56	0.49	0.58	0.52	0.56	0.44	0.44	0.41	0.41	0.53	0.53	0.53	0.52	0.52	0.52	0.52	0.52	0.41	0.41	0.45	0.49	
59	0.56	0.49	0.58	0.52	0.56	0.44	0.44	0.41	0.41	0.53	0.53	0.53	0.52	0.52	0.52	0.52	0.52	0.41	0.41	0.45	0.49	
60	0.34	0.38	0.27	0.27	0.45	0.39	0.39	0.36	0.36	0.48	0.48	0.48	0.53	0.53	0.53	0.53	0.53	0.42	0.40	0.44	0.47	
61	0.34	0.31	0.47	0.47	0.39	0.32	0.32	0.43	0.43	0.28	0.28	0.28	0.27	0.27	0.27	0.27	0.27	0.36	0.36	0.33	0.38	
62	0.38	0.41	0.19	0.25	0.36	0.30	0.30	0.27	0.27	0.58	0.58	0.58	0.56	0.56	0.56	0.56	0.56	0.51	0.51	0.63	0.53	
63	0.49	0.53	0.38	0.44	0.55	0.49	0.49	0.47	0.47	0.52	0.52	0.52	0.50	0.50	0.50	0.50	0.50	0.51	0.51	0.56	0.53	
64	0.49	0.47	0.38	0.38	0.55	0.49	0.49	0.47	0.47	0.58	0.58	0.58	0.56	0.56	0.56	0.56	0.56	0.57	0.57	0.63	0.59	
65	0.49	0.53	0.43	0.36	0.48	0.41	0.41	0.39	0.39	0.52	0.52	0.52	0.50	0.50	0.50	0.50	0.50	0.52	0.52	0.50	0.47	
66	0.49	0.53	0.43	0.36	0.48	0.41	0.41	0.39	0.39	0.52	0.52	0.52	0.50	0.50	0.50	0.50	0.50	0.52	0.52	0.50	0.47	
67	0.41	0.45	0.41	0.35	0.47	0.40	0.40	0.37	0.37	0.57	0.57	0.57	0.55	0.55	0.55	0.55	0.55	0.50	0.50	0.55	0.45	
68	0.47	0.52	0.35	0.35	0.47	0.40	0.40	0.37	0.37	0.50	0.50	0.50	0.48	0.48	0.48	0.48	0.48	0.50	0.50	0.55	0.45	
69	0.50	0.55	0.37	0.37	0.50	0.43	0.43	0.40	0.40	0.54	0.54	0.54	0.52	0.52	0.52	0.52	0.52	0.47	0.47	0.52	0.41	
70	0.45	0.43	0.46	0.31	0.44	0.39	0.39	0.49	0.49	0.47	0.47	0.47	0.51	0.51	0.51	0.51	0.51	0.58	0.58	0.51	0.60	
71	0.57	0.62	0.49	0.54	0.63	0.53	0.53	0.46	0.46	0.56	0.56	0.56	0.60	0.60	0.60	0.60	0.60	0.70	0.70	0.60	0.62	
72	0.55	0.60	0.40	0.46	0.61	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.74	0.74	0.60	0.67	
73	0.55	0.60	0.40	0.46	0.61	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.74	0.74	0.63	0.72	
74	0.55	0.60	0.40	0.46	0.61	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.74	0.74	0.63	0.72	
75	0.55	0.60	0.40	0.46	0.61	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.74	0.74	0.63	0.72	
76	0.55	0.60	0.40	0.46	0.61	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.74	0.74	0.63	0.72	
77	0.57	0.62	0.43	0.49	0.63	0.53	0.53	0.46	0.46	0.61	0.61	0.61	0.65	0.65	0.65	0.65	0.65	0.70	0.70	0.65	0.67	
78	0.55	0.60	0.40	0.46	0.61	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.74	0.74	0.63	0.72	
79	0.59	0.63	0.44	0.50	0.65	0.54	0.54	0.47	0.47	0.57	0.57	0.57	0.61	0.61	0.61	0.61	0.61	0.72	0.72	0.60	0.68	
80	0.50	0.54	0.40	0.46	0.56	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.74	0.74	0.63	0.70	
81	0.53	0.57	0.42	0.49	0.59	0.59	0.59	0.52	0.52	0.56	0.56	0.56	0.61	0.61	0.61	0.61	0.61	0.61	0.61	0.65	0.67	
82	0.54	0.59	0.44	0.50	0.61	0.61	0.61	0.53	0.53	0.58	0.58	0.58	0.63	0.63	0.63	0.63	0.63	0.61	0.61	0.55	0.63	
83	0.53	0.57	0.42	0.49	0.59	0.59	0.59	0.52	0.52	0.63	0.63	0.63	0.67	0.67	0.67	0.67	0.67	0.56	0.56	0.57	0.60	
84	0.53	0.57	0.42	0.49	0.59	0.59	0.59	0.52	0.52	0.56	0.56	0.56	0.61	0.61	0.61	0.61	0.61	0.61	0.61	0.55	0.63	
85	0.51	0.56	0.41	0.47	0.57	0.57	0.57	0.50	0.50	0.61	0.61	0.61	0.65	0.65	0.65	0.65	0.65	0.60	0.60	0.59	0.61	
86	0.51	0.56	0.41	0.47	0.57	0.51	0.51	0.50	0.50	0.61	0.61	0.61	0.65	0.65	0.65	0.65	0.65	0.60	0.60	0.59	0.61	
87	0.53	0.57	0.42	0.49	0.59	0.59	0.59	0.52	0.52	0.56	0.56	0.56	0.61	0.61	0.61	0.61	0.61	0.61	0.61	0.55	0.63	
88	0.51	0.56	0.41	0.47	0.57	0.57	0.57	0.50	0.50	0.55	0.55	0.55	0.59	0.59	0.59	0.59	0.59	0.65	0.65	0.59	0.67	
89	0.51	0.56	0.41	0.47	0.57	0.57	0.57	0.50	0.50	0.61	0.61	0.61	0.65	0.65	0.65	0.65	0.65	0.60	0.60	0.59	0.61	
90	0.54	0.59	0.44	0.50	0.61	0.61	0.61	0.53	0.53	0.58	0.58	0.58	0.63	0.63	0.63	0.63	0.63	0.57	0.57	0.56	0.59	
91	0.54	0.59	0.44	0.50	0.61	0.61	0.61	0.53	0.53	0.58	0.58	0.58	0.63	0.63	0.63	0.63	0.63	0.57	0.57	0.56	0.59	

Similarity matrix for Trifolium accessions

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
92	0.54	0.58	0.39	0.44	0.60	0.54	0.54	0.47	0.47	0.63	0.63	0.63	0.67	0.67	0.67	0.67	0.67	0.72	0.72	0.67	0.68	0.75
93	0.54	0.58	0.39	0.44	0.60	0.54	0.54	0.47	0.47	0.63	0.63	0.63	0.67	0.67	0.67	0.67	0.67	0.72	0.72	0.67	0.68	0.75
94	0.55	0.60	0.40	0.46	0.61	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.68	0.68	0.68	0.65	0.67
95	0.54	0.58	0.39	0.44	0.60	0.54	0.54	0.47	0.47	0.63	0.63	0.63	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.61	0.63	0.70
96	0.59	0.63	0.44	0.50	0.65	0.54	0.54	0.47	0.47	0.57	0.57	0.57	0.61	0.61	0.61	0.61	0.61	0.72	0.72	0.61	0.68	0.70
97	0.55	0.60	0.40	0.46	0.61	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.74	0.74	0.63	0.70	0.72
98	0.59	0.63	0.44	0.50	0.65	0.54	0.54	0.47	0.47	0.57	0.57	0.57	0.61	0.61	0.61	0.61	0.61	0.72	0.72	0.61	0.68	0.70
99	0.55	0.60	0.40	0.46	0.61	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.74	0.74	0.63	0.70	0.72
100	0.59	0.63	0.44	0.50	0.65	0.54	0.54	0.47	0.47	0.57	0.57	0.57	0.61	0.61	0.61	0.61	0.61	0.72	0.72	0.61	0.68	0.70
101	0.55	0.60	0.40	0.46	0.61	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.74	0.74	0.63	0.70	0.72
102	0.55	0.60	0.40	0.46	0.61	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.74	0.74	0.63	0.70	0.72
103	0.59	0.63	0.44	0.50	0.65	0.54	0.54	0.47	0.47	0.57	0.57	0.57	0.61	0.61	0.61	0.61	0.61	0.72	0.72	0.61	0.68	0.70
104	0.59	0.63	0.44	0.50	0.65	0.54	0.54	0.47	0.47	0.57	0.57	0.57	0.61	0.61	0.61	0.61	0.61	0.72	0.72	0.61	0.68	0.70
105	0.57	0.62	0.43	0.49	0.63	0.53	0.53	0.46	0.46	0.61	0.61	0.61	0.65	0.65	0.65	0.65	0.65	0.70	0.70	0.65	0.67	0.73
106	0.55	0.60	0.40	0.46	0.61	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.74	0.74	0.63	0.65	0.67
107	0.56	0.61	0.41	0.47	0.63	0.57	0.57	0.50	0.50	0.67	0.67	0.67	0.71	0.71	0.71	0.71	0.71	0.72	0.72	0.67	0.68	0.70
108	0.59	0.63	0.44	0.50	0.65	0.54	0.54	0.47	0.47	0.57	0.57	0.57	0.61	0.61	0.61	0.61	0.61	0.72	0.72	0.61	0.68	0.70
109	0.56	0.61	0.41	0.47	0.63	0.57	0.57	0.50	0.50	0.67	0.67	0.67	0.71	0.71	0.71	0.71	0.71	0.72	0.72	0.67	0.68	0.70
110	0.54	0.58	0.39	0.44	0.60	0.54	0.54	0.47	0.47	0.63	0.63	0.63	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.61	0.63	0.70
111	0.58	0.63	0.42	0.49	0.65	0.59	0.59	0.52	0.52	0.63	0.63	0.63	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.63	0.65	0.67
112	0.60	0.65	0.46	0.51	0.67	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.68	0.68	0.68	0.65	0.67
113	0.55	0.60	0.40	0.46	0.61	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.68	0.68	0.68	0.65	0.67
114	0.55	0.60	0.40	0.46	0.61	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.68	0.68	0.68	0.65	0.67
115	0.62	0.67	0.47	0.53	0.69	0.57	0.57	0.50	0.50	0.61	0.61	0.61	0.65	0.65	0.65	0.65	0.65	0.63	0.63	0.63	0.65	0.67
116	0.58	0.63	0.42	0.49	0.65	0.59	0.59	0.52	0.52	0.63	0.63	0.63	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.61	0.63	0.70
117	0.62	0.67	0.47	0.53	0.69	0.57	0.57	0.50	0.50	0.61	0.61	0.61	0.65	0.65	0.65	0.65	0.65	0.63	0.63	0.63	0.65	0.67
118	0.56	0.61	0.41	0.47	0.63	0.57	0.57	0.50	0.50	0.67	0.67	0.67	0.71	0.71	0.71	0.71	0.71	0.72	0.72	0.67	0.68	0.70
119	0.56	0.61	0.41	0.47	0.63	0.57	0.57	0.50	0.50	0.67	0.67	0.67	0.71	0.71	0.71	0.71	0.71	0.72	0.72	0.67	0.68	0.70
120	0.56	0.61	0.41	0.47	0.63	0.57	0.57	0.50	0.50	0.67	0.67	0.67	0.71	0.71	0.71	0.71	0.71	0.72	0.72	0.67	0.68	0.70
121	0.58	0.63	0.42	0.49	0.65	0.59	0.59	0.52	0.52	0.63	0.63	0.63	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.61	0.63	0.70
122	0.58	0.63	0.42	0.49	0.65	0.59	0.59	0.52	0.52	0.63	0.63	0.63	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.61	0.63	0.70
123	0.56	0.61	0.41	0.47	0.63	0.57	0.57	0.50	0.50	0.67	0.67	0.67	0.71	0.71	0.71	0.71	0.71	0.72	0.72	0.67	0.68	0.70
124	0.58	0.63	0.42	0.49	0.65	0.59	0.59	0.52	0.52	0.63	0.63	0.63	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.61	0.63	0.70
125	0.56	0.61	0.41	0.47	0.63	0.57	0.57	0.50	0.50	0.67	0.67	0.67	0.71	0.71	0.71	0.71	0.71	0.72	0.72	0.67	0.68	0.70
126	0.56	0.61	0.41	0.47	0.63	0.57	0.57	0.50	0.50	0.67	0.67	0.67	0.71	0.71	0.71	0.71	0.71	0.72	0.72	0.67	0.68	0.70
127	0.56	0.61	0.41	0.47	0.63	0.57	0.57	0.50	0.50	0.67	0.67	0.67	0.71	0.71	0.71	0.71	0.71	0.72	0.72	0.67	0.68	0.70
128	0.58	0.63	0.42	0.49	0.65	0.59	0.59	0.52	0.52	0.63	0.63	0.63	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.61	0.63	0.70
129	0.58	0.63	0.42	0.49	0.65	0.59	0.59	0.52	0.52	0.63	0.63	0.63	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.61	0.63	0.70
130	0.58	0.63	0.42	0.49	0.65	0.59	0.59	0.52	0.52	0.63	0.63	0.63	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.61	0.63	0.70
131	0.60	0.65	0.46	0.51	0.67	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.68	0.68	0.68	0.65	0.67
132	0.60	0.65	0.46	0.51	0.67	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.68	0.68	0.68	0.65	0.67
133	0.60	0.65	0.46	0.51	0.67	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.68	0.68	0.68	0.65	0.67
134	0.60	0.65	0.46	0.51	0.67	0.56	0.56	0.49	0.49	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.68	0.68	0.68	0.65	0.67

Similarity matrix for Trifolium accessions

	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
1																								
2																								
3																								
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18																								
19																								
20																								
21																								
22	1.00																							
23		1.00																						
24	0.62		1.00																					
25	0.96	0.64	1.00																					
26	0.32	0.42	0.33	1.00																				
27	0.36	0.47	0.37	0.86	1.00																			
28	0.41	0.32	0.43	0.78	0.79	1.00																		
29	0.58	0.46	0.61	0.52	0.57	0.55	1.00																	
30	0.62	0.57	0.64	0.55	0.60	0.55	0.92	1.00																
31	0.43	0.53	0.44	0.46	0.56	0.56	0.44	0.40	1.00															
32	0.35	0.40	0.36	0.40	0.44	0.43	0.56	0.59	0.56	1.00														
33	0.56	0.47	0.52	0.36	0.37	0.46	0.33	0.39	0.42	0.46	1.00													
34	0.39	0.36	0.33	0.37	0.46	0.45	0.44	0.37	0.41	0.39	0.39	1.00												
35	0.44	0.41	0.39	0.47	0.45	0.44	0.44	0.48	0.52	0.46	0.40	0.29	1.00											
36	0.37	0.41	0.39	0.47	0.45	0.44	0.44	0.50	0.42	0.36	0.33	0.24	0.87	1.00										
37	0.29	0.33	0.30	0.34	0.37	0.46	0.47	0.50	0.42	0.36	0.38	0.29	0.49	0.54	1.00									
38	0.29	0.33	0.30	0.34	0.37	0.46	0.47	0.50	0.42	0.36	0.38	0.29	0.49	0.54	0.96	1.00								
39	0.36	0.60	0.37	0.40	0.50	0.42	0.50	0.53	0.56	0.30	0.56	0.46	0.39	0.45	0.47	0.53	1.00							
40	0.57	0.26	0.60	0.29	0.32	0.46	0.57	0.52	0.40	0.30	0.48	0.36	0.25	0.25	0.39	0.45	0.50	1.00						
41	0.33	0.31	0.35	0.39	0.36	0.41	0.50	0.46	0.36	0.44	0.38	0.32	0.37	0.37	0.41	0.47	0.50	0.57	1.00					
42	0.44	0.55	0.46	0.35	0.45	0.38	0.52	0.55	0.52	0.15	0.57	0.53	0.33	0.40	0.32	0.32	0.65	0.42	0.30	1.00				
43	0.52	0.64	0.55	0.40	0.52	0.43	0.61	0.64	0.52	0.18	0.52	0.40	0.39	0.46	0.42	0.42	0.67	0.40	0.35	0.77	1.00			
44	0.37	0.48	0.39	0.29	0.39	0.38	0.52	0.48	0.52	0.15	0.57	0.53	0.27	0.33	0.32	0.38	0.65	0.50	0.37	0.87	0.69	1.00		
45	0.42	0.46	0.44	0.26	0.36	0.41	0.50	0.46	0.43	0.17	0.56	0.52	0.30	0.37	0.35	0.41	0.57	0.48	0.42	0.82	0.70	0.89	1.00	

Similarity matrix for Trifolium accessions

	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
46	0.41	0.56	0.42	0.34	0.42	0.41	0.47	0.50	0.58	0.30	0.57	0.54	0.38	0.38	0.46	0.50	0.68	0.45	0.35	0.70	0.67	0.70	0.59	1.00
47	0.37	0.55	0.39	0.24	0.32	0.38	0.52	0.55	0.65	0.46	0.51	0.41	0.33	0.47	0.54	0.60	0.58	0.42	0.44	0.53	0.54	0.60	0.67	0.63
48	0.36	0.53	0.37	0.29	0.38	0.42	0.50	0.53	0.69	0.44	0.56	0.46	0.32	0.39	0.47	0.53	0.63	0.48	0.43	0.58	0.52	0.65	0.64	0.68
49	0.36	0.53	0.37	0.29	0.38	0.42	0.50	0.53	0.63	0.44	0.50	0.40	0.32	0.39	0.47	0.53	0.56	0.40	0.43	0.53	0.52	0.58	0.64	0.68
50	0.37	0.55	0.39	0.29	0.39	0.44	0.52	0.55	0.65	0.46	0.51	0.41	0.33	0.40	0.49	0.54	0.58	0.42	0.44	0.53	0.54	0.60	0.67	0.65
51	0.43	0.60	0.44	0.34	0.44	0.42	0.50	0.60	0.69	0.44	0.56	0.46	0.39	0.45	0.47	0.47	0.63	0.40	0.36	0.65	0.59	0.58	0.57	0.68
52	0.37	0.55	0.39	0.24	0.32	0.38	0.52	0.55	0.65	0.46	0.46	0.35	0.33	0.47	0.60	0.60	0.52	0.33	0.37	0.53	0.62	0.53	0.59	0.65
53	0.36	0.53	0.37	0.29	0.38	0.42	0.50	0.53	0.63	0.44	0.44	0.34	0.32	0.39	0.53	0.53	0.50	0.32	0.36	0.58	0.59	0.52	0.57	0.68
54	0.54	0.43	0.48	0.24	0.33	0.32	0.62	0.57	0.60	0.32	0.65	0.61	0.35	0.35	0.33	0.33	0.53	0.52	0.39	0.62	0.56	0.62	0.54	0.56
55	0.54	0.43	0.48	0.24	0.33	0.32	0.62	0.57	0.60	0.32	0.65	0.61	0.35	0.35	0.33	0.33	0.53	0.52	0.39	0.62	0.56	0.62	0.54	0.56
56	0.50	0.40	0.52	0.34	0.44	0.55	0.57	0.53	0.50	0.44	0.50	0.34	0.45	0.45	0.42	0.42	0.56	0.56	0.50	0.32	0.37	0.32	0.36	0.42
57	0.39	0.43	0.40	0.42	0.47	0.52	0.69	0.64	0.73	0.56	0.53	0.42	0.41	0.55	0.61	0.61	0.67	0.61	0.54	0.48	0.48	0.46	0.56	0.58
58	0.43	0.53	0.44	0.40	0.50	0.61	0.50	0.53	0.50	0.30	0.44	0.29	0.45	0.45	0.53	0.53	0.63	0.56	0.43	0.52	0.59	0.52	0.50	0.58
59	0.43	0.53	0.44	0.40	0.50	0.61	0.50	0.53	0.50	0.30	0.44	0.29	0.45	0.45	0.53	0.53	0.63	0.56	0.43	0.52	0.59	0.52	0.50	0.58
60	0.37	0.48	0.39	0.29	0.39	0.38	0.52	0.48	0.52	0.39	0.57	0.53	0.33	0.33	0.32	0.38	0.52	0.33	0.44	0.53	0.46	0.60	0.67	0.60
61	0.37	0.41	0.39	0.29	0.32	0.44	0.44	0.48	0.39	0.23	0.46	0.41	0.47	0.47	0.54	0.54	0.39	0.33	0.30	0.60	0.62	0.53	0.59	0.54
62	0.41	0.65	0.43	0.50	0.55	0.41	0.62	0.65	0.55	0.36	0.49	0.39	0.50	0.50	0.51	0.56	0.79	0.39	0.48	0.50	0.57	0.50	0.48	0.56
63	0.41	0.32	0.36	0.44	0.42	0.47	0.55	0.52	0.55	0.36	0.54	0.44	0.50	0.50	0.46	0.46	0.61	0.54	0.48	0.36	0.57	0.56	0.48	0.51
64	0.48	0.58	0.43	0.50	0.55	0.53	0.69	0.71	0.61	0.36	0.60	0.44	0.44	0.44	0.46	0.46	0.67	0.54	0.48	0.36	0.57	0.56	0.48	0.51
65	0.56	0.44	0.58	0.44	0.55	0.53	0.64	0.67	0.62	0.42	0.55	0.44	0.43	0.57	0.46	0.46	0.48	0.46	0.40	0.57	0.67	0.50	0.56	0.51
66	0.56	0.44	0.58	0.44	0.55	0.53	0.64	0.67	0.62	0.42	0.55	0.44	0.43	0.57	0.46	0.46	0.48	0.46	0.40	0.57	0.67	0.50	0.56	0.51
67	0.54	0.50	0.56	0.49	0.60	0.52	0.69	0.71	0.60	0.32	0.53	0.42	0.41	0.48	0.39	0.39	0.53	0.44	0.39	0.62	0.72	0.55	0.54	0.56
68	0.54	0.57	0.56	0.42	0.53	0.52	0.77	0.79	0.73	0.48	0.53	0.36	0.35	0.48	0.50	0.50	0.47	0.44	0.42	0.59	0.55	0.64	0.55	0.50
69	0.50	0.46	0.52	0.39	0.50	0.48	0.75	0.79	0.73	0.48	0.53	0.36	0.35	0.48	0.50	0.50	0.47	0.44	0.42	0.63	0.52	0.57	0.56	0.53
70	0.44	0.47	0.39	0.51	0.50	0.54	0.50	0.53	0.50	0.39	0.60	0.51	0.51	0.57	0.48	0.48	0.61	0.54	0.48	0.63	0.52	0.57	0.56	0.53
71	0.53	0.44	0.49	0.54	0.53	0.62	0.59	0.61	0.63	0.55	0.57	0.49	0.60	0.54	0.55	0.55	0.58	0.52	0.53	0.49	0.42	0.43	0.41	0.59
72	0.50	0.47	0.45	0.56	0.56	0.60	0.63	0.65	0.67	0.52	0.60	0.51	0.51	0.51	0.48	0.48	0.61	0.48	0.50	0.51	0.45	0.46	0.44	0.57
73	0.50	0.47	0.45	0.56	0.56	0.60	0.63	0.65	0.67	0.52	0.60	0.51	0.51	0.51	0.48	0.48	0.61	0.48	0.50	0.51	0.45	0.46	0.44	0.57
74	0.50	0.47	0.45	0.56	0.56	0.60	0.63	0.65	0.67	0.52	0.60	0.51	0.51	0.51	0.48	0.48	0.61	0.48	0.50	0.51	0.45	0.46	0.44	0.57
75	0.50	0.47	0.45	0.56	0.56	0.60	0.63	0.65	0.67	0.52	0.60	0.51	0.51	0.51	0.48	0.48	0.61	0.48	0.50	0.51	0.45	0.46	0.44	0.57
76	0.50	0.47	0.45	0.56	0.56	0.60	0.63	0.65	0.67	0.52	0.60	0.51	0.51	0.51	0.48	0.48	0.61	0.48	0.50	0.51	0.45	0.46	0.44	0.57
77	0.53	0.50	0.49	0.59	0.58	0.62	0.65	0.67	0.68	0.49	0.57	0.49	0.54	0.49	0.50	0.50	0.63	0.52	0.47	0.54	0.49	0.49	0.41	0.64
78	0.50	0.47	0.45	0.56	0.56	0.60	0.63	0.65	0.67	0.52	0.60	0.51	0.51	0.51	0.48	0.48	0.61	0.48	0.50	0.51	0.45	0.46	0.44	0.57
79	0.55	0.46	0.50	0.55	0.54	0.63	0.61	0.63	0.65	0.50	0.59	0.50	0.56	0.50	0.51	0.51	0.60	0.53	0.49	0.50	0.44	0.44	0.42	0.61
80	0.50	0.53	0.45	0.62	0.61	0.60	0.69	0.71	0.78	0.52	0.60	0.56	0.51	0.51	0.43	0.43	0.61	0.41	0.44	0.57	0.52	0.51	0.44	0.61
81	0.53	0.50	0.48	0.54	0.59	0.57	0.67	0.69	0.77	0.55	0.63	0.60	0.55	0.55	0.45	0.45	0.59	0.44	0.40	0.55	0.48	0.49	0.47	0.60
82	0.55	0.52	0.50	0.50	0.61	0.59	0.67	0.69	0.82	0.55	0.63	0.60	0.49	0.49	0.40	0.40	0.65	0.44	0.40	0.61	0.55	0.50	0.48	0.62
83	0.53	0.56	0.48	0.54	0.65	0.57	0.67	0.69	0.77	0.55	0.63	0.60	0.55	0.55	0.45	0.45	0.59	0.44	0.40	0.55	0.48	0.49	0.47	0.60
84	0.53	0.50	0.48	0.54	0.59	0.57	0.67	0.69	0.77	0.55	0.63	0.60	0.55	0.55	0.45	0.45	0.59	0.44	0.40	0.55	0.48	0.49	0.47	0.60
85	0.52	0.55	0.47	0.58	0.63	0.56	0.71	0.73	0.80	0.53	0.62	0.58	0.53	0.53	0.44	0.44	0.63	0.43	0.39	0.59	0.53	0.53	0.45	0.63
86	0.58	0.55	0.53	0.58	0.63	0.56	0.77	0.79	0.74	0.47	0.67	0.58	0.53	0.53	0.49	0.49	0.63	0.50	0.39	0.65	0.60	0.59	0.52	0.63
87	0.53	0.50	0.48	0.54	0.59	0.57	0.67	0.69	0.77	0.55	0.63	0.60	0.55	0.55	0.45	0.45	0.59	0.44	0.40	0.55	0.48	0.49	0.47	0.60
88	0.52	0.49	0.47	0.58	0.57	0.61	0.65	0.67	0.74	0.53	0.62	0.58	0.53	0.53	0.44	0.44	0.57	0.43	0.45	0.53	0.47	0.47	0.45	0.59
89	0.52	0.55	0.47	0.58	0.63	0.56	0.71	0.73	0.80	0.53	0.62	0.58	0.53	0.53	0.44	0.44	0.63	0.43	0.39	0.59	0.53	0.53	0.45	0.63
90	0.55	0.52	0.50	0.50	0.61	0.59	0.62	0.75	0.79	0.57	0.65	0.61	0.50	0.50	0.41	0.41	0.61	0.46	0.41	0.56	0.50	0.50	0.48	0.62
91	0.55	0.52	0.50	0.50	0.61	0.59	0.62	0.65	0.79	0.57	0.65	0.61	0.50	0.50	0.41	0.41	0.61	0.46	0.41	0.56	0.50	0.50	0.48	0.62

Similarity matrix for Tribolium accessions

	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
92	0.49	0.51	0.44	0.60	0.60	0.58	0.67	0.69	0.70	0.50	0.59	0.50	0.50	0.50	0.47	0.47	0.65	0.47	0.49	0.56	0.50	0.50	0.42	0.61
93	0.55	0.57	0.50	0.55	0.60	0.53	0.73	0.74	0.76	0.50	0.63	0.53	0.50	0.50	0.47	0.47	0.65	0.47	0.42	0.61	0.56	0.56	0.49	0.65
94	0.56	0.53	0.52	0.51	0.56	0.54	0.69	0.71	0.72	0.52	0.65	0.56	0.51	0.51	0.48	0.48	0.65	0.48	0.44	0.57	0.52	0.51	0.50	0.62
95	0.55	0.57	0.50	0.55	0.60	0.53	0.73	0.74	0.76	0.50	0.63	0.53	0.50	0.50	0.47	0.47	0.65	0.48	0.44	0.57	0.52	0.56	0.49	0.65
96	0.55	0.46	0.50	0.55	0.54	0.63	0.61	0.63	0.65	0.50	0.59	0.50	0.56	0.50	0.51	0.51	0.60	0.53	0.49	0.50	0.44	0.44	0.42	0.61
97	0.50	0.47	0.45	0.56	0.56	0.60	0.63	0.65	0.67	0.52	0.60	0.51	0.51	0.51	0.48	0.48	0.61	0.48	0.50	0.51	0.44	0.46	0.44	0.57
98	0.55	0.46	0.50	0.55	0.54	0.63	0.61	0.63	0.65	0.50	0.59	0.50	0.56	0.50	0.51	0.51	0.60	0.53	0.49	0.50	0.44	0.44	0.42	0.61
99	0.50	0.47	0.45	0.56	0.56	0.60	0.63	0.65	0.67	0.52	0.60	0.51	0.51	0.51	0.48	0.48	0.61	0.48	0.50	0.51	0.44	0.46	0.44	0.57
100	0.55	0.46	0.50	0.55	0.54	0.63	0.61	0.63	0.65	0.50	0.59	0.50	0.56	0.50	0.51	0.51	0.60	0.53	0.49	0.50	0.44	0.44	0.42	0.61
101	0.50	0.47	0.45	0.56	0.56	0.60	0.63	0.65	0.67	0.52	0.60	0.51	0.51	0.51	0.48	0.48	0.61	0.48	0.50	0.51	0.45	0.46	0.44	0.57
102	0.50	0.47	0.45	0.56	0.56	0.60	0.63	0.65	0.67	0.52	0.60	0.51	0.51	0.51	0.48	0.48	0.61	0.48	0.50	0.51	0.45	0.46	0.44	0.57
103	0.55	0.46	0.50	0.55	0.54	0.63	0.61	0.63	0.65	0.50	0.59	0.50	0.56	0.50	0.51	0.51	0.60	0.53	0.49	0.50	0.44	0.44	0.42	0.61
104	0.55	0.46	0.50	0.55	0.54	0.63	0.61	0.63	0.65	0.50	0.59	0.50	0.56	0.50	0.51	0.51	0.60	0.53	0.49	0.50	0.44	0.44	0.42	0.61
105	0.53	0.50	0.49	0.59	0.58	0.62	0.65	0.67	0.68	0.49	0.57	0.49	0.54	0.49	0.50	0.50	0.63	0.52	0.47	0.54	0.49	0.49	0.41	0.64
106	0.56	0.59	0.52	0.56	0.61	0.54	0.75	0.77	0.78	0.52	0.65	0.56	0.51	0.51	0.48	0.48	0.67	0.48	0.44	0.63	0.58	0.57	0.50	0.67
107	0.52	0.55	0.47	0.58	0.63	0.56	0.71	0.73	0.74	0.53	0.62	0.53	0.53	0.53	0.49	0.49	0.69	0.50	0.45	0.59	0.53	0.53	0.45	0.63
108	0.61	0.51	0.56	0.55	0.54	0.63	0.67	0.69	0.70	0.50	0.63	0.55	0.56	0.50	0.51	0.51	0.60	0.53	0.49	0.56	0.50	0.50	0.49	0.65
109	0.52	0.55	0.47	0.58	0.63	0.56	0.71	0.73	0.74	0.53	0.62	0.53	0.53	0.53	0.49	0.49	0.69	0.50	0.45	0.59	0.53	0.53	0.45	0.63
110	0.55	0.57	0.50	0.60	0.60	0.58	0.73	0.74	0.76	0.50	0.63	0.55	0.50	0.50	0.47	0.47	0.65	0.52	0.47	0.55	0.48	0.49	0.47	0.60
111	0.53	0.50	0.48	0.54	0.59	0.57	0.67	0.69	0.71	0.55	0.63	0.54	0.55	0.55	0.50	0.50	0.65	0.52	0.47	0.55	0.48	0.49	0.47	0.60
112	0.56	0.47	0.52	0.51	0.56	0.60	0.63	0.65	0.67	0.52	0.60	0.51	0.57	0.51	0.52	0.52	0.61	0.55	0.44	0.51	0.45	0.46	0.44	0.62
113	0.56	0.53	0.52	0.56	0.56	0.60	0.69	0.71	0.72	0.52	0.65	0.56	0.51	0.51	0.48	0.48	0.61	0.48	0.50	0.57	0.52	0.51	0.50	0.62
114	0.56	0.59	0.52	0.56	0.61	0.54	0.75	0.77	0.78	0.52	0.65	0.56	0.51	0.51	0.48	0.48	0.67	0.48	0.44	0.63	0.58	0.47	0.45	0.63
115	0.58	0.49	0.53	0.53	0.57	0.61	0.65	0.67	0.69	0.53	0.62	0.53	0.55	0.55	0.50	0.50	0.65	0.52	0.47	0.55	0.48	0.49	0.47	0.60
116	0.53	0.50	0.48	0.54	0.59	0.57	0.67	0.69	0.71	0.55	0.63	0.54	0.55	0.55	0.50	0.50	0.65	0.52	0.47	0.55	0.48	0.49	0.47	0.60
117	0.58	0.49	0.53	0.53	0.57	0.61	0.65	0.67	0.69	0.53	0.62	0.53	0.59	0.53	0.49	0.49	0.69	0.50	0.45	0.59	0.53	0.53	0.45	0.63
118	0.52	0.55	0.47	0.58	0.63	0.56	0.71	0.73	0.74	0.53	0.62	0.53	0.53	0.53	0.49	0.49	0.69	0.50	0.45	0.59	0.53	0.53	0.45	0.63
119	0.52	0.55	0.47	0.58	0.63	0.56	0.71	0.73	0.74	0.53	0.62	0.53	0.53	0.53	0.49	0.49	0.69	0.50	0.45	0.59	0.53	0.53	0.45	0.63
120	0.52	0.55	0.47	0.58	0.63	0.56	0.71	0.73	0.74	0.53	0.62	0.53	0.53	0.53	0.49	0.49	0.69	0.50	0.45	0.59	0.53	0.53	0.45	0.63
121	0.53	0.50	0.48	0.54	0.59	0.57	0.67	0.69	0.71	0.55	0.63	0.54	0.55	0.55	0.50	0.50	0.65	0.52	0.47	0.55	0.48	0.49	0.47	0.60
122	0.53	0.50	0.48	0.54	0.59	0.57	0.67	0.69	0.71	0.55	0.63	0.54	0.55	0.55	0.50	0.50	0.65	0.52	0.47	0.55	0.48	0.49	0.47	0.60
123	0.58	0.49	0.53	0.58	0.63	0.61	0.71	0.73	0.73	0.69	0.69	0.69	0.69	0.69	0.49	0.49	0.63	0.50	0.45	0.53	0.47	0.47	0.45	0.59
124	0.53	0.50	0.48	0.54	0.59	0.57	0.67	0.69	0.71	0.55	0.63	0.54	0.55	0.55	0.50	0.50	0.65	0.52	0.47	0.55	0.48	0.49	0.47	0.60
125	0.52	0.49	0.47	0.53	0.57	0.56	0.65	0.67	0.69	0.53	0.62	0.53	0.53	0.53	0.49	0.49	0.63	0.50	0.45	0.53	0.47	0.47	0.45	0.59
126	0.52	0.49	0.47	0.53	0.57	0.56	0.65	0.67	0.69	0.53	0.62	0.53	0.53	0.53	0.49	0.49	0.63	0.50	0.45	0.53	0.47	0.47	0.45	0.59
127	0.52	0.49	0.47	0.53	0.57	0.56	0.65	0.67	0.69	0.53	0.62	0.53	0.53	0.53	0.49	0.49	0.63	0.50	0.45	0.53	0.47	0.47	0.45	0.59
128	0.53	0.50	0.48	0.54	0.59	0.57	0.67	0.69	0.71	0.55	0.63	0.54	0.55	0.55	0.50	0.50	0.65	0.52	0.47	0.55	0.48	0.49	0.47	0.60
129	0.53	0.50	0.48	0.54	0.59	0.57	0.67	0.69	0.71	0.55	0.63	0.54	0.55	0.55	0.50	0.50	0.65	0.52	0.47	0.55	0.48	0.49	0.47	0.60
130	0.53	0.50	0.48	0.54	0.59	0.57	0.67	0.69	0.71	0.55	0.63	0.54	0.55	0.55	0.50	0.50	0.65	0.52	0.47	0.55	0.48	0.49	0.47	0.60
131	0.56	0.47	0.52	0.51	0.56	0.60	0.63	0.65	0.67	0.52	0.60	0.51	0.57	0.51	0.52	0.52	0.61	0.55	0.44	0.51	0.45	0.46	0.44	0.62
132	0.56	0.47	0.52	0.51	0.56	0.60	0.63	0.65	0.67	0.52	0.60	0.51	0.57	0.51	0.52	0.52	0.61	0.55	0.44	0.51	0.45	0.46	0.44	0.62
133	0.56	0.47	0.52	0.51	0.56	0.60	0.63	0.65	0.67	0.52	0.60	0.51	0.57	0.51	0.52	0.52	0.61	0.55	0.44	0.51	0.45	0.46	0.44	0.62
134	0.56	0.47	0.52	0.51	0.56	0.60	0.63	0.65	0.67	0.52	0.60	0.51	0.57	0.51	0.52	0.52	0.61	0.55	0.44	0.51	0.45	0.46	0.44	0.62

Similarity matrix for *Trifolium* accessions

	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
46																								
47	1.00																							
48	0.90	1.00																						
49	0.90		1.00																					
50	0.93	0.97	0.97	1.00																				
51	0.84	0.94	0.88	0.90	1.00																			
52	0.93	0.84	0.84	0.87	0.84	1.00																		
53	0.84	0.88	0.94	0.90	0.88	0.90	1.00																	
54	0.48	0.53	0.47	0.48	0.53	0.48	0.47	1.00																
55	0.48	0.53	0.47	0.48	0.53	0.48	0.47	1.00	1.00															
56	0.45	0.44	0.44	0.45	0.44	0.45	0.44	0.53	0.53	1.00														
57	0.69	0.67	0.60	0.62	0.67	0.69	0.60	0.60	0.57	0.57	1.00													
58	0.52	0.56	0.50	0.52	0.56	0.58	0.56	0.40	0.40	0.63	0.60	1.00												
59	0.52	0.56	0.50	0.52	0.56	0.58	0.56	0.40	0.40	0.63	0.60	1.00	1.00											
60	0.60	0.58	0.58	0.60	0.60	0.52	0.53	0.52	0.41	0.41	0.45	0.48	0.45	0.45	1.00									
61	0.47	0.45	0.52	0.47	0.45	0.53	0.58	0.35	0.35	0.35	0.32	0.41	0.58	0.58	0.47	1.00								
62	0.56	0.55	0.55	0.56	0.56	0.55	0.50	0.49	0.43	0.45	0.55	0.58	0.49	0.49	0.63	0.38	1.00							
63	0.44	0.55	0.55	0.50	0.55	0.55	0.44	0.55	0.38	0.58	0.55	0.71	0.73	0.73	0.50	0.56	0.59	1.00						
64	0.50	0.55	0.49	0.50	0.50	0.55	0.50	0.49	0.38	0.58	0.55	0.71	0.73	0.73	0.50	0.56	0.59	0.65	1.00					
65	0.64	0.55	0.55	0.57	0.62	0.64	0.55	0.52	0.52	0.52	0.48	0.67	0.48	0.48	0.50	0.43	0.47	0.47	0.47	1.00				
66	0.64	0.55	0.55	0.57	0.62	0.64	0.55	0.52	0.52	0.52	0.48	0.67	0.48	0.48	0.50	0.43	0.47	0.47	0.47	1.00				
67	0.55	0.53	0.53	0.55	0.60	0.55	0.53	0.57	0.57	0.57	0.47	0.57	0.47	0.47	0.48	0.41	0.52	0.45	0.52	0.89	1.00			
68	0.69	0.60	0.60	0.62	0.60	0.69	0.60	0.60	0.57	0.57	0.47	0.64	0.53	0.53	0.55	0.48	0.52	0.45	0.65	0.74	0.74	0.79	1.00	
69	0.67	0.57	0.57	0.59	0.57	0.67	0.57	0.62	0.62	0.62	0.43	0.69	0.43	0.43	0.52	0.44	0.48	0.48	0.55	0.80	0.80	0.85	0.92	1.00
70	0.40	0.50	0.44	0.46	0.56	0.40	0.44	0.53	0.53	0.53	0.50	0.59	0.56	0.56	0.46	0.51	0.54	0.70	0.65	0.42	0.42	0.41	0.41	0.38
71	0.49	0.58	0.53	0.54	0.63	0.49	0.53	0.56	0.56	0.56	0.63	0.72	0.58	0.58	0.49	0.43	0.56	0.67	0.67	0.51	0.51	0.50	0.50	0.47
72	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.59	0.59	0.59	0.61	0.77	0.56	0.56	0.51	0.34	0.60	0.70	0.70	0.55	0.55	0.53	0.53	0.50
73	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.59	0.59	0.59	0.61	0.77	0.56	0.56	0.51	0.34	0.60	0.70	0.70	0.55	0.55	0.53	0.53	0.50
74	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.59	0.59	0.59	0.61	0.77	0.56	0.56	0.51	0.34	0.60	0.70	0.70	0.55	0.55	0.53	0.53	0.50
75	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.59	0.59	0.59	0.61	0.77	0.56	0.56	0.51	0.34	0.60	0.70	0.70	0.55	0.55	0.53	0.53	0.50
76	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.59	0.59	0.59	0.61	0.77	0.56	0.56	0.51	0.34	0.60	0.70	0.70	0.55	0.55	0.53	0.53	0.50
77	0.49	0.58	0.53	0.54	0.63	0.49	0.53	0.61	0.61	0.63	0.63	0.72	0.58	0.58	0.49	0.38	0.62	0.67	0.72	0.51	0.51	0.56	0.56	0.53
78	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.59	0.59	0.59	0.61	0.77	0.56	0.56	0.51	0.34	0.60	0.70	0.70	0.55	0.55	0.53	0.53	0.50
79	0.50	0.60	0.54	0.56	0.65	0.50	0.54	0.57	0.57	0.57	0.65	0.74	0.60	0.60	0.50	0.39	0.58	0.68	0.68	0.53	0.53	0.51	0.51	0.49
80	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.65	0.65	0.65	0.50	0.71	0.50	0.50	0.51	0.40	0.65	0.65	0.70	0.55	0.55	0.59	0.59	0.56
81	0.55	0.65	0.59	0.61	0.71	0.55	0.59	0.63	0.63	0.63	0.53	0.75	0.53	0.53	0.55	0.42	0.63	0.69	0.69	0.58	0.58	0.56	0.56	0.53
82	0.56	0.67	0.61	0.63	0.73	0.56	0.61	0.65	0.65	0.65	0.55	0.71	0.55	0.55	0.56	0.38	0.59	0.65	0.65	0.60	0.60	0.58	0.58	0.55
83	0.55	0.65	0.59	0.61	0.71	0.55	0.59	0.69	0.69	0.69	0.53	0.69	0.53	0.53	0.55	0.36	0.63	0.63	0.69	0.58	0.58	0.63	0.63	0.60
84	0.55	0.65	0.59	0.61	0.71	0.55	0.59	0.63	0.63	0.63	0.53	0.75	0.53	0.53	0.55	0.42	0.63	0.69	0.69	0.58	0.58	0.56	0.56	0.53
85	0.53	0.63	0.57	0.59	0.69	0.53	0.57	0.67	0.67	0.67	0.51	0.73	0.51	0.51	0.53	0.41	0.67	0.67	0.72	0.56	0.56	0.61	0.61	0.58
86	0.53	0.63	0.57	0.59	0.69	0.53	0.57	0.73	0.73	0.73	0.51	0.73	0.51	0.51	0.53	0.41	0.67	0.72	0.72	0.63	0.63	0.67	0.67	0.65
87	0.55	0.65	0.59	0.61	0.71	0.55	0.59	0.63	0.63	0.63	0.53	0.75	0.53	0.53	0.55	0.42	0.63	0.69	0.69	0.58	0.58	0.56	0.56	0.53
88	0.53	0.63	0.57	0.59	0.69	0.53	0.57	0.61	0.61	0.61	0.51	0.73	0.51	0.51	0.53	0.41	0.67	0.67	0.72	0.56	0.56	0.61	0.61	0.58
89	0.53	0.63	0.57	0.59	0.69	0.53	0.57	0.67	0.67	0.67	0.51	0.73	0.51	0.51	0.53	0.41	0.67	0.72	0.72	0.56	0.56	0.61	0.61	0.58
90	0.56	0.67	0.61	0.63	0.73	0.56	0.61	0.65	0.65	0.65	0.55	0.71	0.55	0.55	0.56	0.38	0.59	0.65	0.65	0.60	0.60	0.58	0.58	0.55
91	0.56	0.67	0.61	0.63	0.73	0.56	0.61	0.65	0.65	0.65	0.55	0.71	0.55	0.55	0.56	0.38	0.59	0.65	0.65	0.60	0.60	0.58	0.58	0.55

Similarity matrix for Trifolium accessions

	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
92	0.50	0.60	0.54	0.56	0.65	0.50	0.54	0.63	0.63	0.60	0.74	0.54	0.54	0.50	0.33	0.63	0.68	0.74	0.53	0.53	0.57	0.57	0.55
93	0.56	0.60	0.60	0.61	0.70	0.56	0.60	0.69	0.69	0.60	0.74	0.54	0.54	0.56	0.39	0.68	0.68	0.74	0.59	0.59	0.63	0.63	0.61
94	0.57	0.67	0.61	0.63	0.72	0.57	0.61	0.65	0.65	0.61	0.77	0.56	0.56	0.57	0.40	0.65	0.70	0.70	0.61	0.61	0.59	0.59	0.56
95	0.56	0.65	0.60	0.61	0.70	0.56	0.60	0.69	0.69	0.60	0.74	0.54	0.54	0.56	0.39	0.68	0.68	0.74	0.59	0.59	0.63	0.63	0.61
96	0.50	0.60	0.54	0.56	0.65	0.50	0.54	0.57	0.57	0.65	0.74	0.60	0.60	0.50	0.39	0.58	0.68	0.68	0.53	0.53	0.51	0.51	0.49
97	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.59	0.59	0.61	0.77	0.56	0.60	0.50	0.39	0.58	0.68	0.68	0.53	0.53	0.51	0.51	0.49
98	0.50	0.60	0.54	0.56	0.65	0.50	0.54	0.57	0.57	0.65	0.74	0.60	0.60	0.50	0.39	0.58	0.68	0.68	0.53	0.53	0.51	0.51	0.49
99	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.59	0.59	0.61	0.77	0.56	0.60	0.50	0.39	0.58	0.68	0.68	0.53	0.53	0.51	0.51	0.49
100	0.50	0.60	0.54	0.56	0.65	0.50	0.54	0.57	0.57	0.65	0.74	0.60	0.60	0.50	0.39	0.58	0.68	0.68	0.53	0.53	0.51	0.51	0.49
101	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.59	0.59	0.61	0.77	0.56	0.60	0.50	0.39	0.58	0.68	0.68	0.53	0.53	0.51	0.51	0.49
102	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.59	0.59	0.61	0.77	0.56	0.60	0.50	0.39	0.58	0.68	0.68	0.53	0.53	0.51	0.51	0.49
103	0.50	0.60	0.54	0.56	0.65	0.50	0.54	0.57	0.57	0.65	0.74	0.60	0.60	0.50	0.39	0.58	0.68	0.68	0.53	0.53	0.51	0.51	0.49
104	0.50	0.60	0.54	0.56	0.65	0.50	0.54	0.57	0.57	0.65	0.74	0.60	0.60	0.50	0.39	0.58	0.68	0.68	0.53	0.53	0.51	0.51	0.49
105	0.49	0.58	0.53	0.54	0.63	0.49	0.53	0.61	0.61	0.63	0.72	0.58	0.58	0.49	0.40	0.70	0.70	0.76	0.61	0.61	0.65	0.65	0.63
106	0.57	0.67	0.61	0.63	0.72	0.57	0.61	0.71	0.71	0.56	0.77	0.56	0.56	0.57	0.40	0.70	0.70	0.76	0.61	0.61	0.65	0.65	0.63
107	0.53	0.63	0.57	0.59	0.69	0.53	0.57	0.67	0.67	0.57	0.79	0.57	0.57	0.53	0.35	0.67	0.72	0.78	0.59	0.59	0.57	0.57	0.55
108	0.56	0.65	0.60	0.61	0.70	0.56	0.60	0.63	0.63	0.60	0.74	0.60	0.60	0.53	0.35	0.67	0.72	0.78	0.59	0.59	0.57	0.57	0.55
109	0.53	0.63	0.57	0.59	0.69	0.53	0.57	0.67	0.67	0.57	0.79	0.57	0.57	0.53	0.35	0.67	0.72	0.78	0.59	0.59	0.57	0.57	0.55
110	0.56	0.65	0.60	0.61	0.70	0.56	0.60	0.69	0.69	0.54	0.74	0.54	0.54	0.56	0.39	0.68	0.68	0.74	0.59	0.59	0.57	0.57	0.55
111	0.55	0.65	0.59	0.61	0.71	0.55	0.59	0.63	0.63	0.59	0.81	0.59	0.59	0.55	0.36	0.63	0.74	0.74	0.58	0.58	0.56	0.56	0.53
112	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.59	0.59	0.67	0.77	0.61	0.61	0.51	0.40	0.60	0.70	0.70	0.55	0.55	0.53	0.53	0.50
113	0.57	0.67	0.61	0.63	0.72	0.57	0.61	0.71	0.71	0.56	0.77	0.56	0.56	0.57	0.40	0.65	0.70	0.70	0.61	0.61	0.65	0.65	0.63
114	0.57	0.67	0.61	0.63	0.72	0.57	0.61	0.71	0.71	0.56	0.77	0.56	0.56	0.57	0.40	0.65	0.70	0.70	0.61	0.61	0.65	0.65	0.63
115	0.53	0.63	0.57	0.59	0.69	0.53	0.57	0.61	0.61	0.63	0.79	0.63	0.63	0.53	0.41	0.61	0.72	0.72	0.56	0.56	0.55	0.55	0.52
116	0.55	0.65	0.59	0.61	0.71	0.55	0.59	0.63	0.63	0.59	0.81	0.59	0.59	0.55	0.36	0.63	0.74	0.74	0.58	0.58	0.56	0.56	0.53
117	0.53	0.63	0.57	0.59	0.69	0.53	0.57	0.61	0.61	0.63	0.79	0.63	0.63	0.53	0.41	0.61	0.72	0.72	0.56	0.56	0.55	0.55	0.52
118	0.53	0.63	0.57	0.59	0.69	0.53	0.57	0.67	0.67	0.57	0.79	0.57	0.57	0.53	0.35	0.67	0.72	0.78	0.56	0.56	0.61	0.61	0.58
119	0.53	0.63	0.57	0.59	0.69	0.53	0.57	0.67	0.67	0.57	0.79	0.57	0.57	0.53	0.35	0.67	0.72	0.78	0.56	0.56	0.61	0.61	0.58
120	0.53	0.63	0.57	0.59	0.69	0.53	0.57	0.67	0.67	0.57	0.79	0.57	0.57	0.53	0.35	0.67	0.72	0.78	0.56	0.56	0.61	0.61	0.58
121	0.55	0.65	0.59	0.61	0.71	0.55	0.59	0.63	0.63	0.59	0.81	0.59	0.59	0.55	0.36	0.63	0.74	0.74	0.58	0.58	0.56	0.56	0.53
122	0.55	0.65	0.59	0.61	0.71	0.55	0.59	0.63	0.63	0.59	0.81	0.59	0.59	0.55	0.36	0.63	0.74	0.74	0.58	0.58	0.56	0.56	0.53
123	0.47	0.57	0.51	0.53	0.63	0.47	0.51	0.67	0.67	0.67	0.73	0.57	0.57	0.47	0.41	0.61	0.72	0.72	0.56	0.56	0.61	0.61	0.58
124	0.55	0.65	0.59	0.61	0.71	0.55	0.59	0.63	0.63	0.59	0.81	0.59	0.59	0.55	0.36	0.63	0.74	0.74	0.58	0.58	0.56	0.56	0.53
125	0.53	0.63	0.57	0.59	0.69	0.53	0.57	0.61	0.61	0.63	0.79	0.63	0.63	0.53	0.35	0.61	0.72	0.72	0.56	0.56	0.55	0.55	0.52
126	0.53	0.63	0.57	0.59	0.69	0.53	0.57	0.61	0.61	0.63	0.79	0.63	0.63	0.53	0.35	0.61	0.72	0.72	0.56	0.56	0.55	0.55	0.52
127	0.53	0.63	0.57	0.59	0.69	0.53	0.57	0.61	0.61	0.63	0.79	0.63	0.63	0.53	0.35	0.61	0.72	0.72	0.56	0.56	0.55	0.55	0.52
128	0.55	0.65	0.59	0.61	0.71	0.55	0.59	0.63	0.63	0.59	0.81	0.59	0.59	0.55	0.36	0.63	0.74	0.74	0.58	0.58	0.56	0.56	0.53
129	0.55	0.65	0.59	0.61	0.71	0.55	0.59	0.63	0.63	0.59	0.81	0.59	0.59	0.55	0.36	0.63	0.74	0.74	0.58	0.58	0.56	0.56	0.53
130	0.55	0.65	0.59	0.61	0.71	0.55	0.59	0.63	0.63	0.59	0.81	0.59	0.59	0.55	0.36	0.63	0.74	0.74	0.58	0.58	0.56	0.56	0.53
131	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.59	0.59	0.67	0.77	0.61	0.61	0.51	0.40	0.60	0.70	0.70	0.55	0.55	0.53	0.53	0.50
132	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.59	0.59	0.67	0.77	0.61	0.61	0.51	0.40	0.60	0.70	0.70	0.55	0.55	0.53	0.53	0.50
133	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.59	0.59	0.67	0.77	0.61	0.61	0.51	0.40	0.60	0.70	0.70	0.55	0.55	0.53	0.53	0.50
134	0.51	0.61	0.56	0.57	0.67	0.51	0.56	0.59	0.59	0.67	0.77	0.61	0.61	0.51	0.40	0.60	0.70	0.70	0.55	0.55	0.53	0.53	0.50

Similarity matrix for Trifolium accessions

	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92
46																							
47																							
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67																							
68																							
69																							
70	1.00																						
71	0.86	1.00																					
72	0.85	0.95	1.00																				
73	0.85	0.95	1.00	1.00																			
74	0.85	0.95	1.00	1.00	1.00																		
75	0.85	0.95	1.00	1.00	1.00	1.00																	
76	0.85	0.95	1.00	1.00	1.00	1.00	1.00																
77	0.81	0.96	1.00	1.00	1.00	1.00	0.95	1.00															
78	0.85	0.95	1.00	1.00	1.00	1.00	0.98	0.95	1.00														
79	0.83	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	1.00													
80	0.75	0.86	0.90	0.90	0.90	0.90	0.90	0.91	0.90	0.88	1.00												
81	0.74	0.85	0.90	0.90	0.90	0.90	0.90	0.85	0.90	0.87	0.95	1.00											
82	0.70	0.82	0.87	0.87	0.87	0.87	0.87	0.82	0.87	0.84	0.92	0.97	1.00										
83	0.68	0.80	0.84	0.84	0.84	0.84	0.84	0.85	0.84	0.82	0.95	0.94	0.97	1.00									
84	0.74	0.85	0.90	0.90	0.90	0.90	0.90	0.85	0.90	0.87	0.95	1.00	0.97	0.94	1.00								
85	0.72	0.83	0.87	0.87	0.87	0.87	0.87	0.88	0.87	0.85	0.97	0.97	0.94	0.97	0.97	1.00							
86	0.77	0.83	0.87	0.87	0.87	0.87	0.87	0.88	0.87	0.85	0.92	0.92	0.89	0.92	0.92	0.95	1.00						
87	0.74	0.85	0.90	0.90	0.90	0.90	0.90	0.85	0.90	0.87	0.95	1.00	0.97	0.94	1.00	0.97	0.92	1.00					
88	0.77	0.88	0.92	0.92	0.92	0.92	0.92	0.88	0.92	0.90	0.97	0.97	0.94	0.92	0.97	0.95	0.90	0.97	1.00				
89	0.72	0.83	0.87	0.87	0.87	0.87	0.87	0.88	0.87	0.85	0.97	0.97	0.94	0.97	0.97	1.00	0.95	0.97	0.97	1.00			
90	0.70	0.82	0.87	0.87	0.87	0.87	0.87	0.82	0.87	0.84	0.92	0.97	1.00	0.97	0.97	0.94	0.89	0.97	0.97	0.94	1.00		
91	0.70	0.82	0.87	0.87	0.87	0.87	0.87	0.82	0.87	0.84	0.92	0.97	1.00	0.97	0.97	0.94	0.89	0.97	0.97	0.94	1.00	1.00	

Similarity matrix for Trifolium accessions

	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92
92	0.83	0.93	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.95	0.93	0.87	0.84	0.87	0.87	0.90	0.90	0.87	0.90	0.90	0.84	0.84	1.00
93	0.78	0.88	0.93	0.93	0.93	0.93	0.93	0.93	0.93	0.91	0.93	0.92	0.90	0.92	0.92	0.95	0.95	0.92	0.90	0.95	0.90	0.90	0.95
94	0.80	0.91	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.91	0.90	0.95	0.90	0.90	0.95	0.92	0.92	0.95	0.92	0.92	0.90	0.90	0.93
95	0.78	0.88	0.93	0.93	0.93	0.93	0.93	0.93	0.93	0.91	0.90	0.95	0.90	0.90	0.95	0.92	0.92	0.95	0.92	0.90	0.95	0.90	0.95
96	0.83	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	1.00	0.88	0.87	0.84	0.82	0.87	0.85	0.85	0.87	0.90	0.85	0.84	0.84	0.95
97	0.85	0.95	1.00	1.00	1.00	1.00	1.00	0.95	1.00	0.98	0.90	0.90	0.87	0.84	0.90	0.87	0.87	0.90	0.92	0.87	0.87	0.87	0.98
98	0.83	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	1.00	0.88	0.87	0.84	0.82	0.87	0.85	0.85	0.87	0.90	0.85	0.84	0.84	0.95
99	0.85	0.95	1.00	1.00	1.00	1.00	1.00	0.95	1.00	0.98	0.90	0.90	0.87	0.84	0.90	0.87	0.87	0.90	0.92	0.87	0.87	0.87	0.98
100	0.83	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	1.00	0.88	0.87	0.84	0.82	0.87	0.85	0.85	0.87	0.90	0.85	0.84	0.84	0.95
101	0.85	0.95	1.00	1.00	1.00	1.00	1.00	0.95	1.00	0.98	0.90	0.90	0.87	0.84	0.90	0.87	0.87	0.90	0.92	0.87	0.87	0.87	0.98
102	0.85	0.95	1.00	1.00	1.00	1.00	1.00	0.95	1.00	0.98	0.90	0.90	0.87	0.84	0.90	0.87	0.87	0.90	0.92	0.87	0.87	0.87	0.98
103	0.83	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	1.00	0.88	0.87	0.84	0.82	0.87	0.85	0.85	0.87	0.90	0.85	0.84	0.84	0.95
104	0.83	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	1.00	0.88	0.87	0.84	0.82	0.87	0.85	0.85	0.87	0.90	0.85	0.84	0.84	0.95
105	0.81	0.96	0.95	0.95	0.95	0.95	0.95	1.00	0.95	0.98	0.91	0.85	0.82	0.85	0.85	0.88	0.88	0.85	0.88	0.88	0.82	0.82	0.98
106	0.75	0.86	0.90	0.90	0.90	0.90	0.90	0.91	0.90	0.88	0.95	0.95	0.92	0.95	0.95	0.97	0.97	0.95	0.92	0.97	0.92	0.92	0.93
107	0.77	0.88	0.92	0.92	0.92	0.92	0.92	0.93	0.92	0.90	0.92	0.92	0.89	0.92	0.92	0.95	0.95	0.92	0.90	0.95	0.89	0.89	0.95
108	0.77	0.88	0.92	0.92	0.92	0.92	0.92	0.93	0.92	0.90	0.92	0.92	0.89	0.92	0.92	0.95	0.95	0.92	0.90	0.95	0.89	0.89	0.95
109	0.77	0.88	0.92	0.92	0.92	0.92	0.92	0.93	0.92	0.90	0.92	0.92	0.89	0.92	0.92	0.95	0.95	0.92	0.90	0.95	0.89	0.89	0.95
110	0.78	0.88	0.93	0.93	0.93	0.93	0.93	0.93	0.93	0.91	0.98	0.92	0.90	0.92	0.92	0.95	0.95	0.92	0.90	0.95	0.90	0.90	0.95
111	0.79	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92	0.92	0.94	0.92	0.92	0.91	0.91	0.92
112	0.80	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.98	0.85	0.90	0.87	0.84	0.90	0.87	0.87	0.90	0.87	0.87	0.87	0.87	0.93
113	0.80	0.91	0.95	0.95	0.95	0.95	0.95	0.91	0.95	0.93	0.95	0.95	0.92	0.90	0.95	0.92	0.92	0.95	0.92	0.92	0.92	0.92	0.93
114	0.75	0.86	0.90	0.90	0.90	0.90	0.90	0.91	0.90	0.88	0.95	0.95	0.92	0.95	0.95	0.97	0.97	0.95	0.92	0.97	0.92	0.92	0.93
115	0.77	0.93	0.92	0.92	0.92	0.92	0.92	0.93	0.92	0.95	0.87	0.92	0.89	0.87	0.92	0.90	0.90	0.92	0.90	0.90	0.89	0.89	0.90
116	0.79	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92	0.92	0.94	0.92	0.92	0.91	0.91	0.92
117	0.77	0.93	0.92	0.92	0.92	0.92	0.92	0.93	0.92	0.95	0.87	0.92	0.89	0.87	0.92	0.90	0.90	0.92	0.90	0.90	0.89	0.89	0.90
118	0.77	0.88	0.92	0.92	0.92	0.92	0.92	0.93	0.92	0.90	0.92	0.92	0.89	0.92	0.92	0.95	0.95	0.92	0.90	0.95	0.89	0.89	0.95
119	0.77	0.88	0.92	0.92	0.92	0.92	0.92	0.93	0.92	0.90	0.92	0.92	0.89	0.92	0.92	0.95	0.95	0.92	0.90	0.95	0.89	0.89	0.95
120	0.77	0.88	0.92	0.92	0.92	0.92	0.92	0.93	0.92	0.90	0.92	0.92	0.89	0.92	0.92	0.95	0.95	0.92	0.90	0.95	0.89	0.89	0.95
121	0.79	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92	0.92	0.94	0.92	0.90	0.89	0.89	0.95
122	0.79	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92	0.92	0.94	0.92	0.90	0.89	0.89	0.95
123	0.77	0.88	0.87	0.87	0.87	0.87	0.87	0.93	0.87	0.90	0.87	0.87	0.83	0.87	0.84	0.90	0.95	0.87	0.84	0.90	0.83	0.83	0.90
124	0.79	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92	0.92	0.94	0.92	0.92	0.91	0.91	0.92
125	0.82	0.93	0.97	0.97	0.97	0.97	0.97	0.93	0.97	0.95	0.87	0.92	0.89	0.87	0.92	0.90	0.90	0.92	0.90	0.90	0.89	0.89	0.95
126	0.82	0.93	0.97	0.97	0.97	0.97	0.97	0.93	0.97	0.95	0.87	0.92	0.89	0.87	0.92	0.90	0.90	0.92	0.90	0.90	0.89	0.89	0.95
127	0.82	0.93	0.97	0.97	0.97	0.97	0.97	0.93	0.97	0.95	0.87	0.92	0.89	0.87	0.92	0.90	0.90	0.92	0.90	0.90	0.89	0.89	0.95
128	0.79	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92	0.92	0.94	0.92	0.92	0.91	0.91	0.92
129	0.79	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92	0.92	0.94	0.92	0.92	0.91	0.91	0.92
130	0.79	0.90	0.95	0.95	0.95	0.95	0.95	0.90	0.95	0.92	0.90	0.94	0.91	0.89	0.94	0.92	0.92	0.94	0.92	0.92	0.91	0.91	0.92
131	0.80	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.98	0.85	0.90	0.87	0.84	0.90	0.87	0.87	0.90	0.87	0.87	0.87	0.87	0.93
132	0.80	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.98	0.85	0.90	0.87	0.84	0.90	0.87	0.87	0.90	0.87	0.87	0.87	0.87	0.93
133	0.80	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.98	0.85	0.90	0.87	0.84	0.90	0.87	0.87	0.90	0.87	0.87	0.87	0.87	0.93
134	0.80	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.98	0.85	0.90	0.87	0.84	0.90	0.87	0.87	0.90	0.87	0.87	0.87	0.87	0.93

Similarity matrix for Tridolum accessions

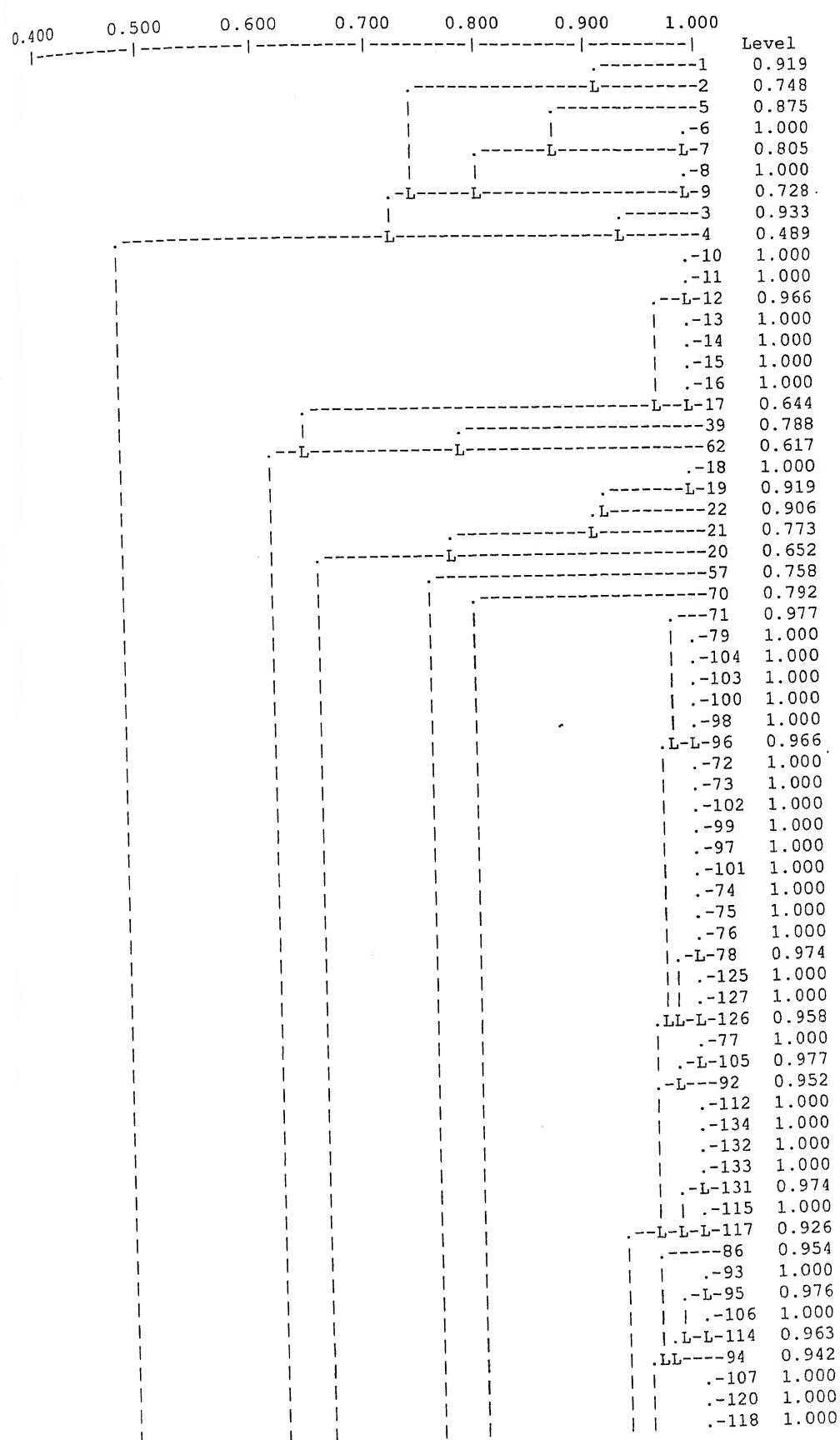
	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115
92																								
93	1.00																							
94	0.98	1.00																						
95	1.00	0.98	1.00																					
96	0.91	0.93	0.91	1.00																				
97	0.93	0.95	0.93	0.98	1.00																			
98	0.91	0.93	0.91	1.00	0.98	1.00																		
99	0.93	0.95	0.93	0.98	1.00	0.98	1.00																	
100	0.91	0.93	0.91	1.00	0.98	1.00	0.98	1.00																
101	0.93	0.95	0.93	0.98	1.00	0.98	1.00	0.98	1.00															
102	0.93	0.95	0.93	0.98	1.00	0.98	1.00	0.98	1.00	1.00														
103	0.91	0.93	0.91	1.00	0.98	1.00	0.98	1.00	0.98	0.98	1.00													
104	0.91	0.93	0.91	1.00	0.98	1.00	0.98	1.00	0.98	0.98	1.00	1.00												
105	0.93	0.95	0.93	0.98	1.00	0.98	1.00	0.98	0.95	0.95	0.95	0.98	0.88	1.00										
106	0.98	0.95	0.93	0.98	0.88	0.90	0.88	0.90	0.88	0.90	0.90	0.88	0.91	0.91	1.00									
107	0.95	0.92	0.95	0.90	0.90	0.92	0.90	0.92	0.90	0.92	0.92	0.90	0.90	0.93	0.97	1.00								
108	0.91	0.93	0.91	0.95	0.93	0.93	0.93	0.95	0.93	0.93	0.93	0.95	0.95	0.93	0.93	0.90	1.00							
109	0.95	0.92	0.95	0.90	0.92	0.90	0.92	0.92	0.90	0.92	0.92	0.90	0.90	0.93	0.97	1.00	0.90	1.00						
110	0.95	0.93	0.95	0.91	0.93	0.91	0.93	0.93	0.91	0.93	0.93	0.91	0.91	0.93	0.98	0.95	0.95	0.95	1.00					
111	0.92	0.95	0.92	0.92	0.92	0.95	0.92	0.95	0.92	0.95	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00				
112	0.93	0.95	0.93	0.93	0.93	0.95	0.93	0.95	0.93	0.95	0.95	0.93	0.93	0.91	0.95	0.92	0.93	0.92	0.88	0.95	1.00			
113	0.93	0.95	0.93	0.93	0.93	0.95	0.93	0.95	0.93	0.95	0.95	0.93	0.93	0.91	0.95	0.92	0.93	0.92	0.88	0.95	0.90	1.00		
114	0.98	0.95	0.93	0.98	0.88	0.90	0.88	0.90	0.88	0.90	0.90	0.88	0.88	0.91	1.00	0.97	0.93	0.97	0.98	0.95	0.90	0.95	1.00	
115	0.90	0.92	0.90	0.95	0.92	0.95	0.92	0.95	0.92	0.95	0.92	0.95	0.95	0.93	0.92	0.95	0.95	0.95	0.90	0.97	0.97	0.92	0.92	1.00
116	0.92	0.95	0.92	0.92	0.92	0.95	0.92	0.95	0.92	0.95	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00	0.95	0.95	0.95	0.97
117	0.90	0.92	0.90	0.95	0.90	0.92	0.95	0.92	0.95	0.92	0.92	0.95	0.95	0.93	0.92	0.95	0.95	0.95	0.90	0.97	0.97	0.92	0.92	1.00
118	0.95	0.92	0.95	0.90	0.90	0.92	0.90	0.92	0.90	0.92	0.92	0.90	0.90	0.93	0.97	1.00	0.90	1.00	0.95	0.97	0.92	0.92	0.97	0.95
119	0.95	0.92	0.95	0.90	0.90	0.92	0.90	0.92	0.90	0.92	0.92	0.90	0.90	0.93	0.97	1.00	0.90	1.00	0.95	0.97	0.92	0.92	0.97	0.95
120	0.95	0.92	0.95	0.90	0.90	0.92	0.90	0.92	0.90	0.92	0.92	0.90	0.90	0.93	0.97	1.00	0.90	1.00	0.95	0.97	0.92	0.92	0.97	0.95
121	0.92	0.95	0.92	0.92	0.92	0.95	0.92	0.95	0.92	0.95	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00	0.95	0.95	0.95	0.97
122	0.92	0.95	0.92	0.92	0.92	0.95	0.92	0.95	0.92	0.95	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00	0.95	0.95	0.95	0.97
123	0.90	0.87	0.90	0.90	0.90	0.87	0.90	0.87	0.90	0.87	0.87	0.90	0.90	0.93	0.92	0.95	0.90	0.95	0.90	0.92	1.00	0.92	0.87	0.95
124	0.92	0.95	0.92	0.92	0.92	0.95	0.92	0.95	0.92	0.95	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00	0.95	0.95	0.95	0.97
125	0.95	0.97	0.95	0.95	0.95	0.97	0.95	0.97	0.95	0.97	0.97	0.95	0.95	0.93	0.92	0.95	0.90	0.95	0.90	0.97	0.97	0.92	0.92	0.95
126	0.95	0.97	0.95	0.95	0.95	0.97	0.95	0.97	0.95	0.97	0.97	0.95	0.95	0.93	0.92	0.95	0.90	0.95	0.90	0.97	0.97	0.92	0.92	0.95
127	0.95	0.97	0.95	0.95	0.95	0.97	0.95	0.97	0.95	0.97	0.97	0.95	0.95	0.93	0.92	0.95	0.90	0.95	0.90	0.97	0.97	0.92	0.92	0.95
128	0.92	0.95	0.92	0.92	0.92	0.95	0.92	0.95	0.92	0.95	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00	0.95	0.95	0.95	0.97
129	0.92	0.95	0.92	0.92	0.92	0.95	0.92	0.95	0.92	0.95	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00	0.95	0.95	0.95	0.97
130	0.92	0.95	0.92	0.92	0.92	0.95	0.92	0.95	0.92	0.95	0.95	0.92	0.92	0.90	0.95	0.97	0.92	0.97	0.92	1.00	0.95	0.95	0.95	0.97
131	0.93	0.95	0.93	0.98	0.98	0.95	0.98	0.95	0.98	0.95	0.95	0.98	0.98	0.95	0.90	0.92	0.93	0.92	0.88	0.95	1.00	0.90	0.90	0.97
132	0.93	0.95	0.93	0.98	0.98	0.95	0.98	0.95	0.98	0.95	0.95	0.98	0.98	0.95	0.90	0.92	0.93	0.92	0.88	0.95	1.00	0.90	0.90	0.97
133	0.93	0.95	0.93	0.98	0.98	0.95	0.98	0.95	0.98	0.95	0.95	0.98	0.98	0.95	0.90	0.92	0.93	0.92	0.88	0.95	1.00	0.90	0.90	0.97
134	0.93	0.95	0.93	0.98	0.98	0.95	0.98	0.95	0.98	0.95	0.95	0.98	0.98	0.95	0.90	0.92	0.93	0.92	0.88	0.95	1.00	0.90	0.90	0.97

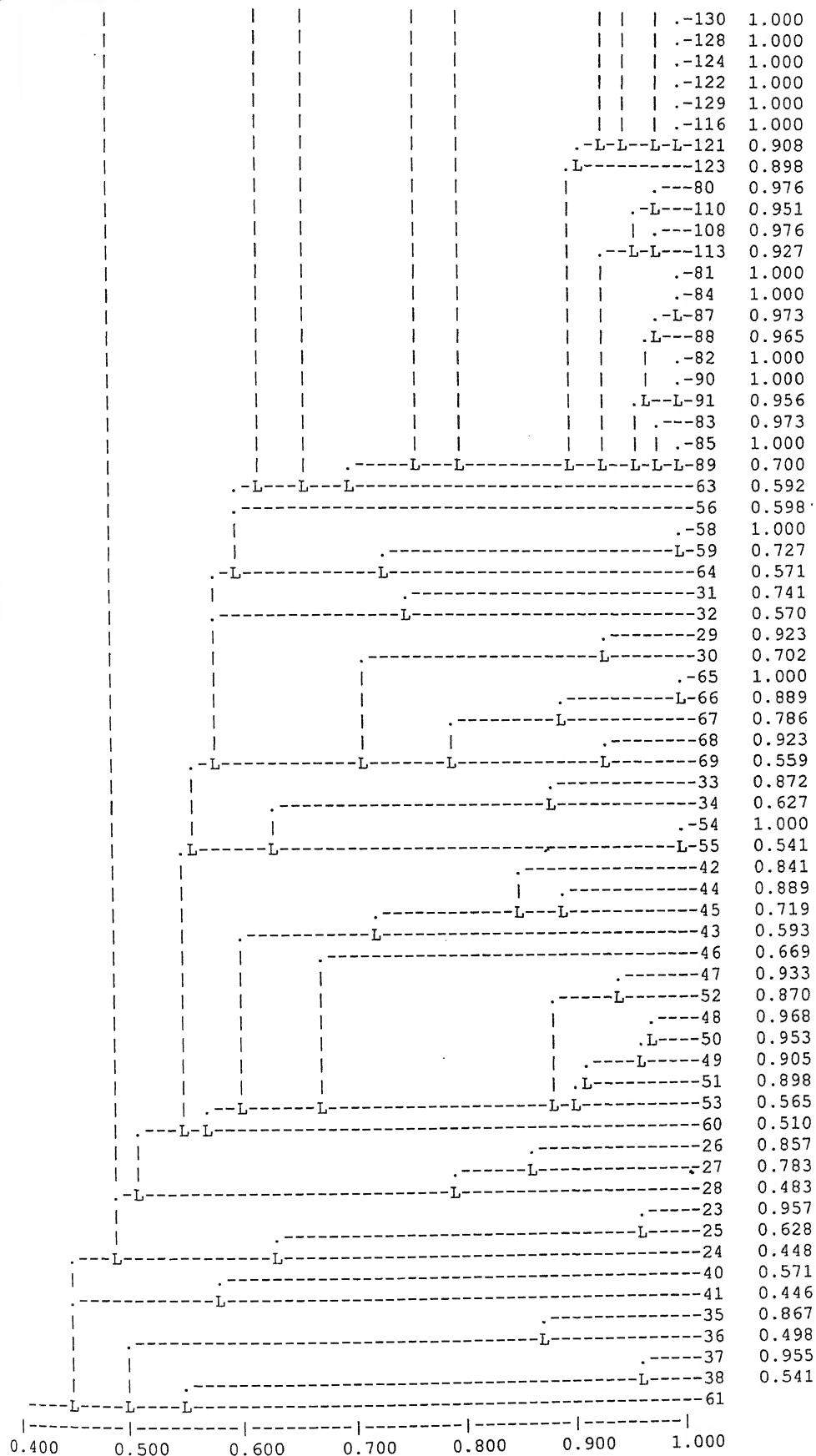
	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134
92																			
93																			
94																			
95																			
96																			
97																			
98																			
99																			
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112																			
113																			
114																			
115																			
116	1.00																		
117	0.97	1.00																	
118	0.97	0.95	1.00																
119	0.97	0.95	1.00	1.00															
120	0.97	0.95	1.00	1.00	1.00														
121	1.00	0.97	0.97	0.97	0.97	1.00													
122	1.00	0.97	0.97	0.97	0.97	0.97	1.00												
123	0.92	0.95	0.95	0.95	0.95	0.92	0.92	1.00											
124	1.00	0.97	0.97	0.97	0.97	0.90	0.90	0.92	1.00										
125	0.97	0.95	0.95	0.95	0.95	0.97	0.97	0.90	0.97	1.00									
126	0.97	0.95	0.95	0.95	0.95	0.97	0.97	0.97	0.90	0.97	1.00								
127	0.97	0.95	0.95	0.95	0.95	0.97	0.97	0.97	0.90	0.97	0.97	1.00							
128	1.00	0.97	0.97	0.97	0.97	1.00	1.00	0.92	1.00	0.97	0.97	0.97	1.00						
129	1.00	0.97	0.97	0.97	0.97	1.00	1.00	0.92	1.00	0.97	0.97	0.97	1.00	1.00					
130	1.00	0.97	0.97	0.97	0.97	1.00	1.00	0.92	1.00	0.97	0.97	0.97	1.00	1.00	1.00				
131	0.95	0.97	0.97	0.92	0.92	0.95	0.95	0.92	0.95	0.97	0.97	0.97	0.95	0.95	0.95	1.00			
132	0.95	0.97	0.97	0.92	0.92	0.95	0.95	0.92	0.95	0.97	0.97	0.97	0.95	0.95	0.95	1.00	1.00		
133	0.95	0.97	0.97	0.92	0.92	0.95	0.95	0.92	0.95	0.97	0.97	0.97	0.95	0.95	0.95	1.00	1.00	1.00	
134	0.95	0.97	0.97	0.92	0.92	0.95	0.95	0.92	0.95	0.97	0.97	0.97	0.95	0.95	0.95	1.00	1.00	1.00	1.00

Table 4.38. Clustering pattern on basis of isozyme analysis in different *Trifolium* species

Cluster No.	Species	No. of accessions
1	<i>T. repens</i>	9
2	<i>T. pratense</i>	8
3	<i>T. cherleri</i> , <i>T. spumosum</i>	1 each
4	<i>T. subterraneum</i>	5
5	<i>T. constantinopolitanum</i>	1
6	<i>T. alexandrinum</i>	65
7	<i>T. tembense</i>	1
8	<i>T. medium</i>	1
9	<i>T. glomeratum</i> , <i>T. apertum</i>	2, 1
10	<i>T. echinatum</i>	2
11	<i>T. alpastre</i>	2
12	<i>T. hybridum</i>	5
13	<i>T. purpureum</i> , <i>T. angustifolium</i>	2 each
14	<i>T. resupinatum</i>	12
15	<i>T. retusum</i>	1
16	<i>T. hirtum</i>	3
17	<i>T. nigrescens</i>	3
18	<i>T. lappaceum</i> , <i>T. diffusum</i>	1 each
19	<i>T. campestre</i> , <i>T. incarnatum</i> , <i>T. argutum</i>	2, 2, 1

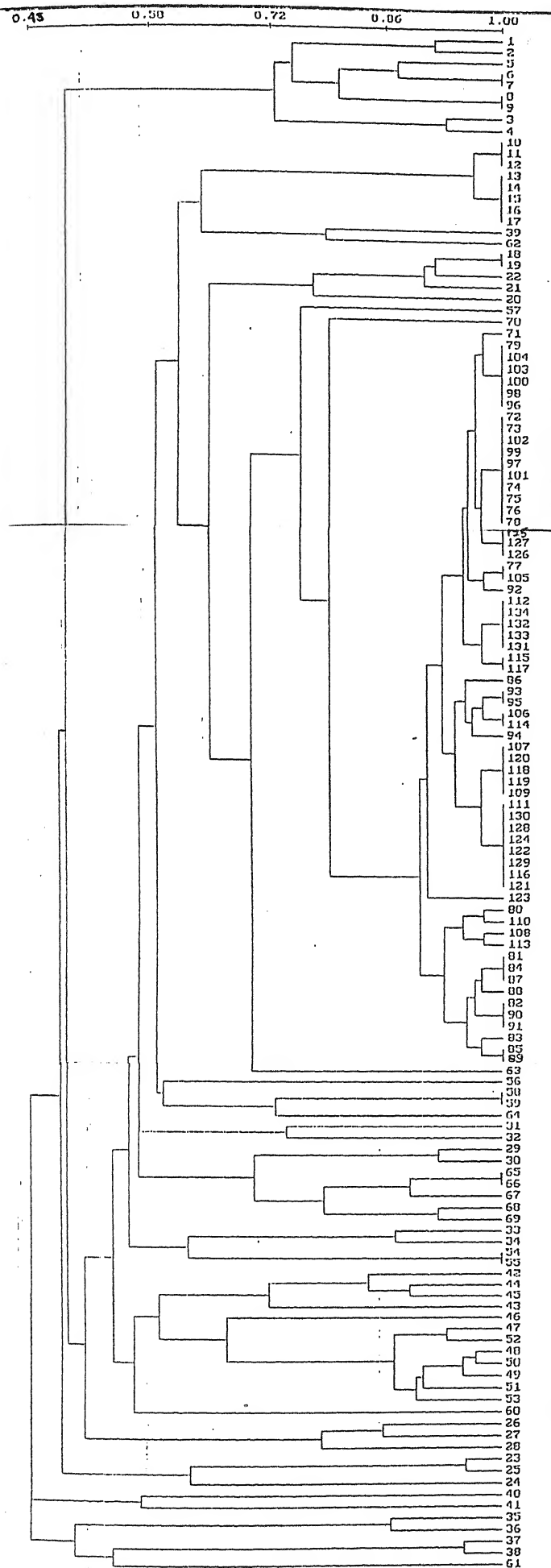
Fig. 24. Clustering based on isozyme data showing genetic relatedness among 134 accessions belonging to 25 *Trifolium* species.





For details of accessions of *Trifolium* species (numbered from 1 to 134) refer to Fig. 25.

Fig 25 : Dendrogram based on cluster analysis of isozyme data showing genetic relatedness among accessions of *Trifolium* species.



Accessions of *Trifolium* species

1-9 *T. repens* (1. EC 401707, 2. EC 401704, 3. EC 401708, 4. EC 401705, 5. EC 401706, 6. EC 400985, 7. EC 400986, 8. EC 400984, EC 400987), 10-17 *T. pratense* (10. EC 400979, 11. PRC-3, 12. EC 400980, 13. EC 400982, 14. EC 401721, 15. EC 401719, 16. EC 401720, 17. EC 400735), 18-22 *T. subterraneum* (18. IC 96-113, 19. IC 96-112, 20. EC 402167, 21. EC 401717, 22. EC 401718), 23-25 *T. nigrescens* (23. EC 425048, 24. EC 425047, 25. EC 425049), 26-28 *T. hirtum* (26. EC 425038, EC 425037, 28. EC 425039), 29-30 *T. alpestre* (29. EC 425042, 30. EC 425043), 31-32 *T. echinatum* (31. EC 401714, 32. EC 405076), 33-34 *T. purpureum* (33. EC 425069, 34. EC 425070), 35-36 *T. campestre* (35. EC 425028, 36. EC 425036), 37-38 *T. incarnatum* (37. IC 96-111, 38. EC 402164), 39 *T. chertleri* (EC 401703), 40 *T. lappaceum* (EC 422165), 41 *T. diffusum* (EC 422163), 42-53 *T. resupinatum* (42. SH 98-36, 43. SH 98-72, 44. SH 98-73, 45. SH 98-86, 46. JHS-3, 47. SH 99-29, 48. SH 99-69, 49. SH 99-33, 50. SH 99-23, 51. SH 99-32, 52. SH 99-25, 53. SH 99-26, 54-55 *T. angustifolium* (54. EC 425062, 55. EC 425061), 56 *T. medium* (EC 425045), 57 *T. constantinopolitanum* (EC 401713), 58-59 *T. glomeratum* (58. EC 402170, 59. EC 401700), 60 *T. retusum* (EC 402150), 61 *T. argutum* (EC 422154), 62 *T. spumosum* (EC 402160), 63 *T. temense* (EC 402169), 64 *T. apertum* (EC 401712), 65-69 *T. hybridum* (65. EC 401702, 66. EC 401701, 67. EC 425032, 68. EC 425029, 69. EC 425030, 70-134 *T. alexandrinum* (70. EC 329299, 71. IL 40010, 72. EC 400733, 73. EC 401710, 74. EC 401709, 75. Warden, 76. EC 402161, 77. EC 400977, 78. EC 400976, 79. EC 401711, 80. JHB 94P-22, 81. JHB94-R-16, 82. JHB94-R-35, 83. JHB94-R-13, 84. JHB94-R-23, 85. JHB94P-T-34, 86. EC 318951, 87. JHB 57P3, 88. JHB P17-1, 89. Raj 7/13-14, 90. JHB 15-27, 91. JHB 6/54 p/t, 92. JHB92-1, 93. BL 122, 94. JHB 146, 95. Raj 7/49-50, 96. JHTB 9-90 N1, 97. JHB 5-13/12, 98. IL 4009, 99. JHTB 5-90-2, 100. JHTB 3-90-H, 101. JHTB 13-90-B, 102. Raj 7/53-54, 103. JHTB-1-90-P3, 104. Raj 7/53-54-O, 105. Raj 7/53-54-2, 106. Raj 7/13-14-O, 107. JHTB 5-90-1, 108. IL 40010-Mes, 109. JHB 94-18/11, 110. JHB 91 P20, 111. JHB 34/22, 112. Raj-Bundi-O, 113. JHB -P-23/35, 114. JHTB -1-90-A1, 115. JHB 94-31, 116. JHB94-25, 117. IL 40014, 118. IL 40013, 119. JHB 94-P-60, 120. BL 144, 121. JHB 94-56, 122. BL 142, 123. Raj 7/13-25, 124. HFB 155, 125. BL 131, 126. JHB 36/5-54, 127. JHB CT2 6/35, 128. JHB 6/54, 129. JHB 16/2, 130. HFB 16/2, 131. Warden S-1, 132. Warden S-2, 133. Warden S-3, 134. Warden S-4.

observed between sub cluster 1 & 2. Cluster no. 2 showed 64.4% similarity with cluster no.3.

In cluster number 3, single accessions each of two species *i.e.* *T. cherleri* and *T. spumosum* were present. *T. spumosum* showed 78.8% similarity with *T. cherleri*. In cluster no. 4, all five accessions of *T. subterraneum* were present. EC 402167 showed 77% similarity with EC 401717 and both showed 91% similarity with EC 401718. 100% similarity was observed between IG 96-112 and IG 96-113. *T. constantinopolitanum* (EC 401713) made separate cluster *i.e.* cluster no. 5 and showed 75.8% similarity with cluster no. 6 comprising of accessions of *T. alexandrinum*.

All 65 accessions of *T. alexandrinum* were present in cluster No 6. Except EC 329299, the rest 64 accessions of *T. alexandrinum* showed >90% similarity among themselves, whereas EC 329299 (a Saidi type) was 79% similar to rest of accessions. Cluster 5 and 6 showed 70% similarity with clusters no.7 (comprising of single accession of *T. tembense*).

T. medium (EC 425045) was present in cluster number 8 and showed 59.8% similarity with cluster no.9. In cluster no. 9, *T. apertum* (EC 401712) showed 73% similarity with two accessions of *T. glomeratum*. Two accessions of *T. glomeratum* (EC 401700 and EC 402170) were 100% similar. In cluster no. 10, two accessions of *T. echinatum* were present and EC 405076 showed 74% similarity with EC 401714.

Two accessions of *T. alpestre* (EC 425042 and EC 425043) made separate cluster (cluster no. 11). The two accessions were 92% similar and this cluster was 70.2% similar with cluster no. 12 of *T. hybridum*. Five accessions of *T. hybridum* were present in cluster no. 12. No difference for isozyme banding pattern was seen between EC 401702 and EC 401701. 92% similarity was observed between EC 425030 and EC 425029 and both were 79% similar to EC 425032. In cluster no. 13, two accessions each of two species *i.e.* *T. purpureum* and *T. angustifolium* were present in two small sub clusters. Eighty seven percent similarity was observed between two accessions of *T. purpureum* *i.e.* EC 425069 and EC 425070 and the two accessions of *T. angustifolium* (EC 425062 and EC 425061) showed 100% similarity. Sixty three percent similarity was observed between *T. purpureum* and *T.*

angustifolium. This cluster showed 54.1% similarity with group of clusters no. 2 to 12. Twelve accessions of *T. resupinatum* made separate cluster and are represented in cluster no. 14. This cluster showed 56.5% similarity with cluster no. 15 of *T. retusum* (EC 402150). In cluster no. 16, three accessions of *T. hirtum* were included. Eighty six percent similarity was observed between EC 425038 and EC 425037 whereas EC 425039 showed 78% similarity with both these accessions. Cluster no. 16 showed 48.3% similarity with group of cluster no. 2 to 15. Cluster no. 1 of *T. repens* showed 48.9% similarity with various species grouped in clusters 2 to 16.

Three accessions of *T. nigrescens* (EC 425048, EC 425049 and EC 425047) were present in cluster number 17. Ninety six percent similarity was observed between EC 425048 and EC 425049 and these together showed 63% similarity with EC 425047. This cluster showed 44.8% similarity with cluster no. 1 to 16.

Single accessions each of *T. lappaceum* and *T. diffusum* were included in cluster No 18. and 57% similarity was observed between these two species. Cluster No. 18 showed 44.6% similarity with cluster no.1 to 17.

In cluster no. 19, three sub clusters were observed. Two accessions of *T. campestre* i.e. EC 425028 and EC 425026 were observed to be 86.9% similar. IG 96-111 and EC 402164 of *T. incarnatum* showed 95.5% similarity. *T. argutum* EC 422154 was 54.1% similar with *T. incarnatum*. This cluster showed 44.6% similarity with remaining cluster i.e. 1 to 18.

4.4. Protein analysis

4.4.1. Qualitative protein estimation

The electrophoretic pattern for leaf protein of 12 species of *Trifolium* was carried out on native PAGE. Electrophoretic bands in these species was recorded and numbered based on relative mobility. The data on banding pattern among different species is presented in Table 4.39. In all, 28 electrophoretic bands were recorded. None of the species showed identical banding pattern. Out of four samples of *T. alexandrinum*, three samples were of self progeny of accession number JHB 99-32 and one of JHB 99-25. The two samples of selfed progeny and that of JHB 99-25 showed identical banding pattern i.e. presence of Band 1, 2, 3, 5, 6, 16, 18, 19, 22, 23, 25, and 27 whereas one plant in the self progeny showed presence of Band

		Protein Banding Pattern in different <i>Trifolium</i> species																											
Band No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Species																													
<i>T. echinatum</i> (EC 425078)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+
<i>T. hybridum</i> (EC 425032)	-	-	+	+	-	+	+	-	+	-	-	-	+	+	-	-	+	+	-	+	-	-	+	-	-	-	-	-	+
<i>T. hirtum</i> (EC 425045)	-	+	+	-	+	+	+	-	+	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-
<i>T. purpureum</i> (EC 425069)	+	+	+	-	+	+	+	+	-	-	-	+	+	-	+	+	+	+	-	-	+	-	+	-	-	+	-	+	-
<i>T. vesiculosum</i> (EC 402168)	+	+	+	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	-	-	-	+	-	-	-	-	-	-	+
<i>T. pratense</i> (EC 401721)	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-	+	-	-	-	-
<i>T. resupinatum</i> (SH 98-15)	+	+	+	-	-	-	+	+	-	-	-	-	-	+	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-
<i>T. repens</i> (EC 400385)	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
<i>T. alexandrinum</i> (JHB 99-32-1)	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	+	+	-	-	-	-	-
<i>T. alexandrinum</i> (JHB 99-32-2)	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	+	-	-	-	-	-
<i>T. alexandrinum</i> (JHB 99-32-3)	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	+	-	-	-	-	-
<i>T. alexandrinum</i> (JHB 99-25)	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	+	-	-	-	-	-
<i>T. angustifolium</i> (EC 425062)	-	-	+	-	-	-	-	+	-	-	+	-	-	-	+	+	+	-	-	+	-	-	-	-	-	-	+	-	-
<i>T. apertum</i> (EC 401712)	+	+	+	-	+	+	+	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	-
<i>T. lappaceum</i> (EC 402165)	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+

**Plate 18 : Variation for leaf protein in different
Trifolium species**

From L to R :

Marker

T. echinatum [EC 425078]

T. hybridum [EC 425032]

T. hirtum [EC 425045]

T. purpureum [EC 425069]

T. vesiculosum [EC 402168]

T. pratense [EC 401721]

T. resupinatum [SH 98-15]

T. repens [EC 400385]

Control

T. alexandrinum [JHB 99-32-1]

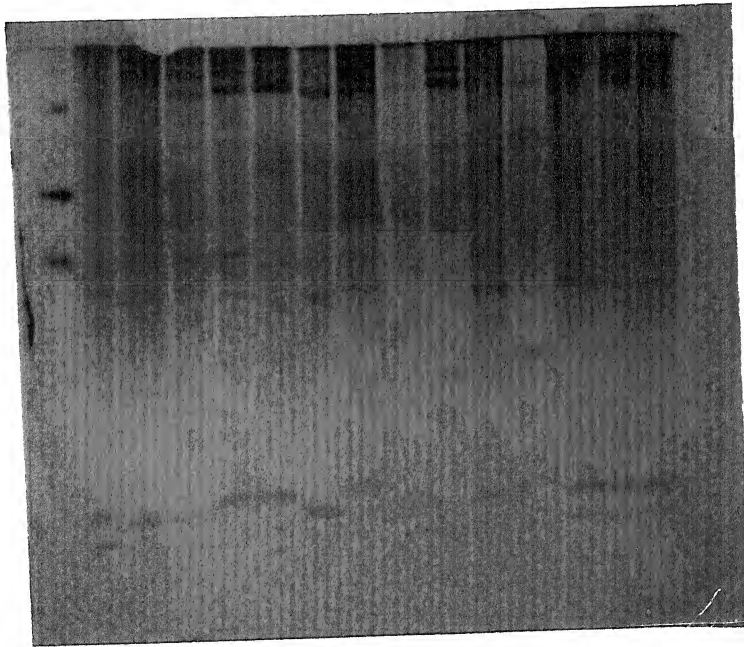
T. angustifolium [EC 425062]

T. alexandrinum [JHB 99-32-2]

T. alexandrinum [JHB 99-32-3]

T. alexandrinum [JHB 99-25]

Plate : 18



number 2, 3, 16, 17, 19, 22, 23, 25, and 27. The maximum number of bands present in any species was 13 in *T. purpureum* (Band 1, 2, 3, 5, 6, 7, 8, 11, 16, 17, 18, 19 and 27) and *T. vesiculosum* (Band 1, 2, 3, 11, 12, 14, 15, 16, 17, 20, 22, 25 and 27) and the minimum bands were found in *T. echinatum* (Band 16, 19, 22, 28) followed with 6 bands in *T. pratense*. Band number 3 was most commonly represented and was found in 11 out of 12 species studies. Band 4 and 9 in *T. hybridum*, Band 10 in *T. angustifolium*, Band 15 in *T. vesiculosum*, Band 23 in *T. alexandrinum* and Band 24 and 26 in *T. resupinatum* were found to be species specific.

The similarity matrix showed the highest degree of similarity among three *T. alexandrinum* accessions out of four studied with *T. apertum* (87% similarity) (Table 4.40). These three *T. alexandrinum* samples together with *T. apertum* showed 70% similarity with the single plant of the selfed *T. alexandrinum* (JHB 99-32-1).

In the clustering by UPGMA, method different species were grouped in total six clusters (Table 4.41). The first cluster comprising of *T. echinatum*, *T. hybridum* and *T. hirtum* showed 34.3% similarity with group of cluster no. 2 to 6 (Fig 26, 27). One accession each of *T. apertum* and *T. purpureum* were clustered with four accessions of *T. alexandrinum* in cluster number 2. The four samples of *T. alexandrinum* along with one *T. apertum* showed 74% similarity with *T. purpureum*. The second cluster was 57.3% similar with cluster number 3 of *T. vesiculosum*. Cluster no. 4 comprised of one accession each of *T. resupinatum*, *T. repens* and *T. lappaceum*. In this cluster *T. resupinatum* and *T. repens* showed 66.7% similarity and together they were 61.3% similar with *T. lappaceum*. This cluster was 52.2% similar with group of cluster 2 and 3. Cluster no. 5 of *T. angustifolium* was 44.2% similar with group of cluster no. 2 to 4. Similarly the group of cluster no. 2 to 5 showed 37.3% similarity with cluster no. 6 of *T. pratense*. Cluster no.1 and the group of clusters 2 to 6 showed 34.3% similarity.

4.4.2 Quantitative crude protein estimation

The genotypes of berseem represented by two major groups i.e. diploid and tetraploid were analysed for crude protein content. Both the groups showed genotypic variations. Crude protein (CP) content varied from 18.88 to 21.32% in diploid and from 19.81 to 22.52 in tetraploid lines (Table 4.42). Almost all the cultivars showed better crude protein content than that of the national check

Table 4.40. Similarity matrix based on protein banding pattern in different <i>Trifolium</i> species														
S.N.	Accessions	1	2	3	4	5	6	7	8	9	10	11	12	15
1	<i>T. echinatum</i> (EC 425078)	1.000												
2	<i>T. hybridum</i> (EC 425032)	0.400	1.000											
3	<i>T. hirtum</i> (EC 425045)	0.429	0.476	1.000										
4	<i>T. purpureum</i> (EC 425069)	0.235	0.417	0.522	1.000									
5	<i>T. vesiculosum</i> (EC 402168)	0.235	0.333	0.435	0.538	1.000								
6	<i>T. pratense</i> (EC 401721)	0.200	0.353	0.375	0.316	0.421	1.000							
7	<i>T. resupinatum</i> (SH 98-15)	0.000	0.273	0.476	0.583	0.333	0.471	1.000						
8	<i>T. repens</i> (EC 400385)	0.000	0.222	0.353	0.600	0.500	0.462	0.667	1.000					
9	<i>T. alexandrinum</i> (JHB 99-32-1)	0.462	0.300	0.421	0.545	0.636	0.267	0.300	0.500	1.000				
10	<i>T. alexandrinum</i> (JHB 99-32-2)	0.375	0.348	0.545	0.720	0.560	0.333	0.435	0.526	0.762	1.000			
11	<i>T. alexandrinum</i> (JHB 99-32-3)	0.375	0.348	0.545	0.720	0.560	0.333	0.435	0.526	0.762	1.000	1.000		
12	<i>T. alexandrinum</i> (JHB 99-25)	0.375	0.348	0.545	0.720	0.560	0.333	0.435	0.526	0.762	1.000	0.500	1.000	
13	<i>T. angustifolium</i> (EC 425062)	0.167	0.316	0.222	0.476	0.381	0.286	0.316	0.400	0.471	0.500	0.870	0.526	1.000
14	<i>T. apertum</i> (EC 401712)	0.267	0.364	0.476	0.833	0.583	0.353	0.545	0.667	0.700	0.870	0.571	0.353	0.700
15	<i>T. lappaceum</i> (EC 402165)	0.308	0.400	0.421	0.727	0.455	0.533	0.600	0.625	0.444	0.571	0.571	0.353	1.000

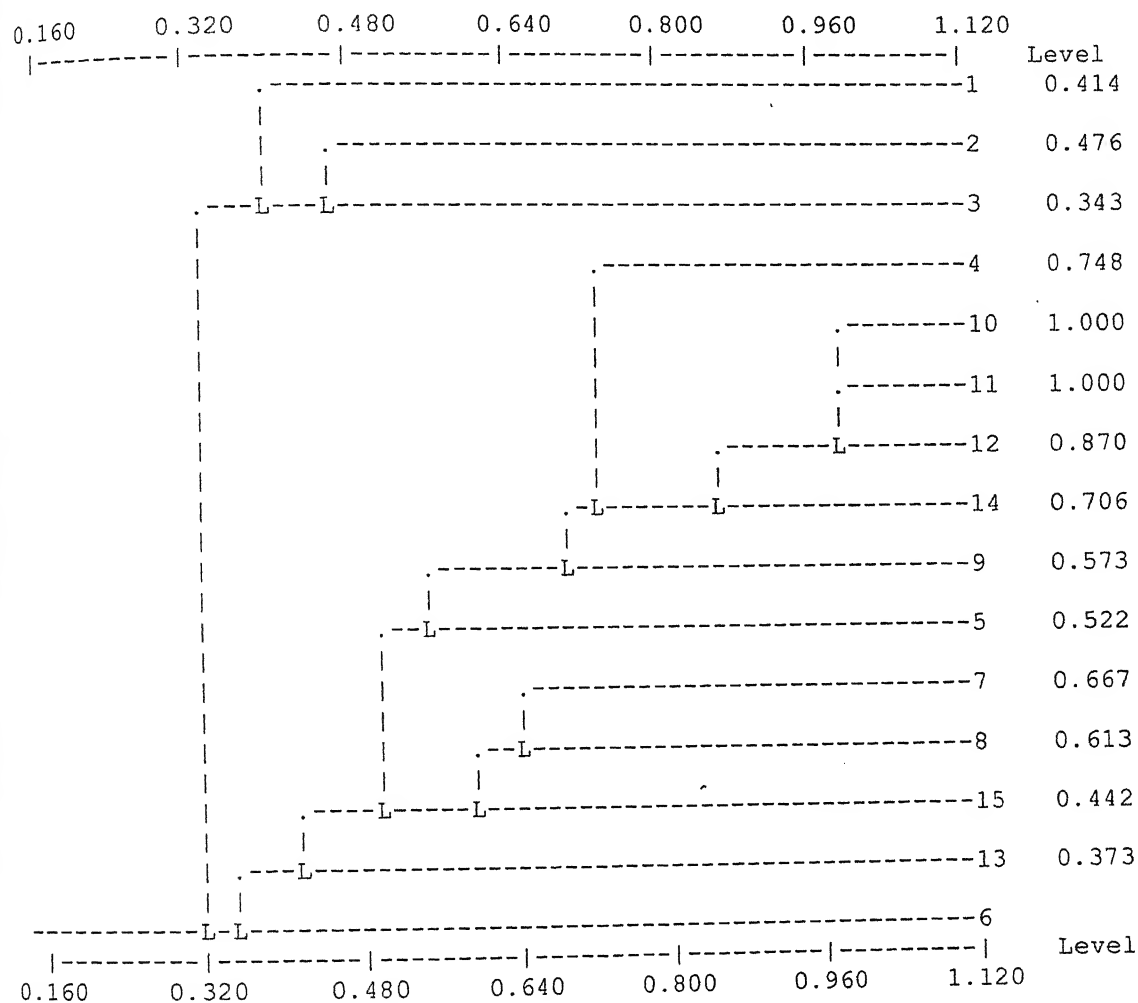
Table 4.41. Clustering of different *Trifolium* species based on leaf protein banding pattern

Cluster No.	Species and accession
1	<i>T. echinatum</i> (EC425078), <i>T. hybridum</i> (EC 425032), <i>T. hirtum</i> (EC 425045)
2	<i>T. purpureum</i> (EC 425069), <i>T. alexandrinum</i> (JHB 99-32-1), <i>T. alexandrinum</i> (JHB 99-32-2), <i>T. alexandrinum</i> (JHB 99-32-3), <i>T. alexandrinum</i> (JHB 99-25), <i>T. apertum</i> (EC 401712)
3	<i>T. vesiculosum</i> (EC 402168)
4	<i>T. resupinatum</i> (EC 401715), <i>T. repens</i> (EC 400385), <i>T. lappaceum</i> (EC 402165)
5	<i>T. angustifolium</i> (EC 425062)
6	<i>T. pratense</i> (EC 401721)

Table 4.42. Crude protein content (% dry matter basis) in different accessions of *Trifolium alexandrinum*.

S.No	Genotype	Ploidy level	CP% (DM basis)
1	EC 329299	2x	20.21
2	JHB 146	2x	20.12
3	JHB 94-18/11	2x	21.32
4	JHB 94-R-25	2x	20.02
5	JHB 57-P3	2x	19.87
6	Raj 7/13-25	2x	19.01
7	Wardan	2x	18.88
8	JHTB 9-90-N1	4x	21.92
9	JHTB 3-90-H	4x	19.81
10	JHTB 1-90A1	4x	22.52

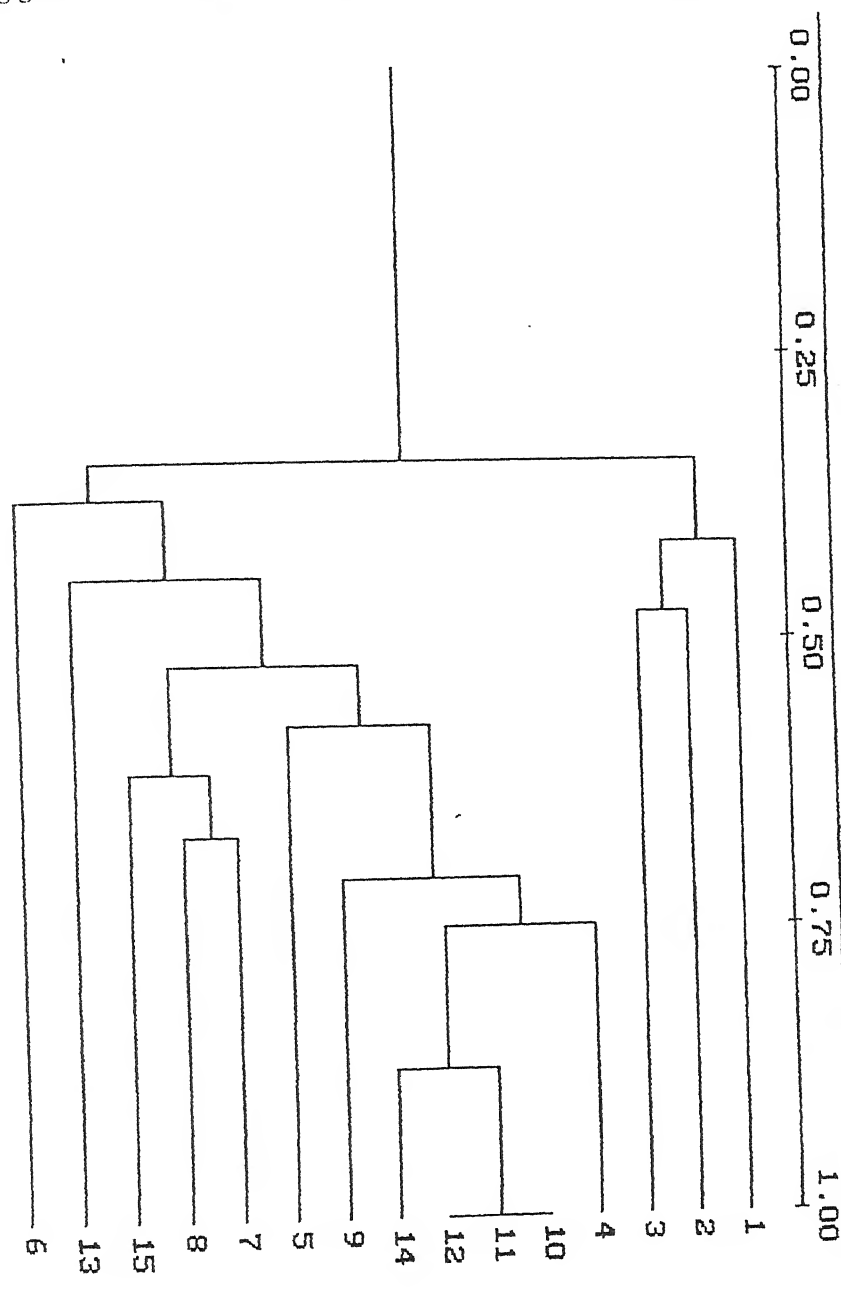
Fig. 26. Clustering based on leaf protein data showing relatedness among different accessions of *Trifolium* species.



List of accessions

1. *T. echinatum* (EC 425078), 2. *T. hybridum* (EC 425032), 3. *T. hirtum* (EC 425045),
4. *T. purpureum* (EC 425069), 5. *T. vesiculosum* (EC 402168), 6. *T. pratense* (EC 401721)
7. *T. resupinatum* (SH 98-15), 8. *T. repens* (EC 400385), 9. *T. alexandrinum* (JHB 99-32-1),
10. *T. alexandrinum* (JHB 99-32-2), 11. *T. alexandrinum* (JHB 99-32-3),
12. *T. alexandrinum* (JHB 99-25), 13. *T. angustifolium* (EC 425062), 14. *T. apertum* (EC 401712),
15. *T. lappaceum* (EC 402165)

Fig 27: Dendrogram based on cluster analysis of leaf protein electrophoresis data showing genetic relatedness between accessions of *Trifolium* species.



Accessions of *Trifolium* species

1. *T. echinatum* (EC 425078), 2. *T. hybridum* (EC 425032), 3. *T. hirtum* (EC 425045),
 4. *T. purpureum* (EC 425069), 5. *T. vesiculosum* (EC 402168), 6. *T. pratense* (EC
 401721), 7. *T. resupinatum* (SH 98-15), 8. *T. repens* (EC 400385), 9. *T. alexandrinum*
 (JHB 99-32-1), 10. *T. alexandrinum* (JHB 99-32-2), 11. *T. alexandrinum* (JHB 99-32-
 3), 12. *T. alexandrinum* (JHB 99-25), 13. *T. angustifolium* (EC 425062), 14. *T.*
apertum (EC 401712), 15. *T. lappaceum* (EC 402165)

Wardan. A few lines, viz JHB 94 -R-25, JHTB 9-90-N1 and JHTB 1-90-A1 showed 2 to 3% higher CP% compared to *Wardan*.

4.5 Interspecific hybridization

Interspecific crosses were attempted for studying the crossability relationship among different species. Flowers were emasculated carefully using eye-lenses and forceps. This was followed by pollination using soft brushes. The suitable time for pollination was found to be 8:00AM. In order to see the efficiency of this crossing method 144 crosses of *T. alexandrinum* x *T. alexandrinum* were made. Forty percent flowers showed no response whereas withering was observed in 59% flowers. Some flowers which showed withering of floral parts were left for seed set under natural condition whereas some other flowers were collected, brought to laboratory and ovary/ovules were removed aseptically under stereoscopic microscope and examined for *in vitro* maturation in hormone free culture media. During embryo rescue healthy ovaries were obtained from 25% flowers out of which 21 ovules were obtained and cultured. Thus, 1.0 ovule per ovary were noticed. Twelve embryos showed good response and got germinated in cultured condition.

Initially the protocol for embryo rescue was developed in intervarietal crosses of *T. alexandrinum*. The standardization of protocol included (i) Appropriate time for embryo excision and (ii) Suitable culture media for embryo germination. In intervarietal controlled crosses of *T. alexandrinum*, ovaries were removed from inflorescence daily from 3 days after pollination (DAP) to 15 DAP to ascertain the optimum time for embryo culture. Ovules and embryos were taken out aseptically and after surface sterilization they were placed in culture media. It was observed that heart shaped embryo was formed at 5 DAP which further developed and attained good size at 9 DAP. Suitable media for ovule and embryo culture was standardized using different hormone combinations. Five media combinations with varying degree of sucrose (2.5 % to 12.5%), NAA (0.006 mg/L to 0.05 mg/L), BAP (2 mg/L to 32 mg/L) were tried to find out appropriate media for ovule and embryo growth. The observations indicated that younger embryo up to 6 DAP require higher sucrose concentration (12.5%) as compared to lower sucrose requirement (2.5%) in case of older and mature embryo (Table 4.43). Interspecific crosses were attempted in many combinations using *T. alexandrinum*

Table 4.43. Stage of ovule/ embryo excision and response

S.N.	Time of excision	Media	Response
1	4 DAP	EC1	No response
		EC2	No response
		EC3	Embryo developed in a few ovules, subcultured, Failed to germinate
		EC4	No response
		EC5	No response
2	6 DAP	EC3	Root developed after 7 days and shoot developed after 11 days
		EC4	No response
		EC 5	No response
3	7 DAP	EC3	Germination of 60% embryos
		EC4	Germination of embryos but checked after 6 to 7 days
		EC5	Germination of embryos checked after 6 to 7 days

DAP= Days after pollination

EC1= L2 basal + 0.001mg/l 2,4 D + 3.225 mg/l adenine + 2.5% sucrose

EC2= L2 basal + 0.006mg/l NAA + 2.0 mg/l adenine + 12.5% sucrose

EC3= MS basal + 0.5 mg/l Kinetin + 3.0% sucrose

EC4= L2 basal + 32.442 mg/ L adenine + 0.0465 mg/L NAA + 12.5% sucrose

EC5= MS basal + 0.5mg/l Kinetin + 12.5% sucrose

as female parent. The post fertilization indicator viz. withering of floral parts, development of ovary were observed in detail. In some of the cases it was observed that although the withering of floral parts such as corolla took place but there was no embryo/ovary development which indicate that the embryos died at very early stage where it was physically not possible to excise the embryo for culturing as embryo could not be differentiated from other maternal tissue. In many other combinations the withering of floral parts was followed by embryo development /ovary elongation. Thus, the embryo abortion differs in different cross combinations which indicate different degree of incompatibility among the species tried in the present study. Details of the various crosses attempted are given in Tables 4.44 and 4.45.

T. alexandrinum x *T. apertum*

A total of four hundred eighty crosses of *T. alexandrinum* x *T. apertum* were attempted and out of these 81.5 % showed no response. Withering of the floral parts was observed only in 18.5% flowers which were used for embryo rescue. During culture, only 8.5% ovaries were noticed to be healthy. Forty one healthy ovaries were obtained, occurrence of one ovule from each ovary was observed. In culture condition, only 4 embryos germinated.

One hundred fifteen cross pollinated flowers of *T. alexandrinum* x *T. apertum* were left in natural condition for seed development but there was no seed formation.

T. alexandrinum x *T. constantinopolitanum*

In this cross combination, 84.2% crosses showed no response while withering of the flower parts was observed in 15.8% crosses. When these flowers were taken for embryo rescue, only 7.4% ovaries were found to be healthy and 33 ovules recovered were cultured in suitable media. On an average 0.7 ovules per ovary were recovered. Only two embryos germinated on culture media.

Fifty six crosses of *T. alexandrinum* and *T. constantinopolitanum* made by emasculation method were left for seed set in natural condition but no seed set was noticed.

Table 4.44. Details of the Interspecific Hybridization in *Trifolium* attempted and used in embryo rescue

SN	Cross	No. of Crosses	Response (%)				Ovules obtained & cultured	Ovules / ovary	Embryo germinated
			No Response (%)	Withering	Healthy ovaries obtained				
1	<i>T. alexandrinum</i> x <i>T. apertum</i>	480	81.5	18.5	8.5	41	1	4	
2	<i>T. alexandrinum</i> x <i>T. constantinopolitanum</i>	612	84.2	15.8	7.4	33	0.7	2	
3	<i>T. alexandrinum</i> x <i>T. echinatum</i>	132	65.9	34	29.5	12	0.3	1	
4	<i>T. alexandrinum</i> x <i>T. repens</i>	144	96.5	3.5	0.69	2	2	-	
5	<i>T. alexandrinum</i> x <i>T. pratense</i>	120	74.2	25.8	15	2	0.11	-	
6	<i>T. alexandrinum</i> x <i>T. resupinatum</i>	340	73.5	26.5	21.2	20	0.28	2	
7	<i>T. alexandrinum</i> x <i>T. incarnatum</i>	43	100	-	-	-	-	-	
8	<i>T. alexandrinum</i> x <i>T. vesiculosum</i>	48	91.6	8.4	4	-	-	-	
9	<i>T. alexandrinum</i> x <i>T. hybridum</i>	50	40.0	60.0	36	-	-	-	
10	<i>T. alexandrinum</i> x <i>T. alexandrinum</i>	144	40	59.02	25	21	1.0	12	
11	<i>T. alexandrinum</i> x <i>T. purpureum</i>	47	100	-	100	94	2.0	0	

Table 4.45. Details of the interspecific hybridization in *Trifolium* attempted and left for seed set

S. No	Cross	No. of Crosses	Seeds obtained
1	<i>T. alexandrinum</i> x <i>T. apertum</i>	115	0
2	<i>T. alexandrinum</i> x <i>T. constantinopolitanum</i>	56	0
3	<i>T. alexandrinum</i> x <i>T. echinatum</i>	140	0
4	<i>T. alexandrinum</i> x <i>T. resupinatum</i>	34	0
5	<i>T. alexandrinum</i> x <i>T. hybridum</i>	65	0
6	<i>T. alexandrinum</i> x <i>T. purpureum</i>	35	0

T. alexandrinum x *T. echinatum*

One hundred thirty two crosses of *T. alexandrinum* x *T. echinatum* were attempted and out of these 65.9 % crosses showed no response. In 34% crosses withering of the flower parts was observed within 2-3 days. For embryo rescue, healthy ovaries were obtained in 29.5% crossed flowers. Only 12 ovules were recovered from 39 ovaries (i.e. 0.3 ovules per ovary), out of which only one embryo germinated.

One hundred forty crosses of *T. alexandrinum* x *T. echinatum* were made using emasculation followed with pollination method and left for hybrid seed set in natural condition. There was no seed formation.

T. alexandrinum x *T. repens*

One hundred forty four crosses of *T. alexandrinum* x *T. repens* were made out of which 96.5% flowers showed no response whereas in 3.5% flowers withering of flower parts was observed. During embryo rescue 0.69% ovaries were found in healthy condition. Only two ovules were obtained from single ovary. These ovules did not germinate in culture condition. It showed that there was no fertilization.

T. alexandrinum x *T. pratense*

One hundred twenty crosses of *T. alexandrinum* x *T. pratense* were attempted and no response was observed in 74.2 % crosses whereas withering was noticed in 25.8% flowers. During embryo rescue 15% ovaries were obtained in healthy condition out of which only 2 ovules could be obtained and cultured on suitable media. The ovules did not germinate in culture condition.

T. alexandrinum x *T. resupinatum*

Three hundred forty crosses of *T. alexandrinum* x *T. resupinatum* were made. Out of these 73.5% flowers showed no response whereas withering was observed in 26.5% flowers. For embryo rescue healthy ovaries were obtained from 21.2% flowers. Twenty ovules were obtained and cultured. Only two embryos germinated in vitro condition.

Thirty four crosses of *T. alexandrinum* x *T. resupinatum* were made using emasculation followed with pollination method and there was no seed formation in natural condition.

T. alexandrinum x *T. incarnatum*

Forty three crosses of *T. alexandrinum* x *T. incarnatum* were attempted, but withering was not noticed in any flowers and no seed set was observed.

T. alexandrinum x *T. vesiculosum*

Out of 48 crosses of *T. alexandrinum* x *T. vesiculosum* made, 91.6% flowers showed no response. Withering was noticed only in 8.4% flowers. Of the total flowers pollinated only 4% ovaries were recorded in good condition. No healthy ovules were obtained.

T. alexandrinum x *T. hybridum*

Fifty crosses of *T. alexandrinum* x *T. hybridum* were attempted and out of these 40% crosses showed no response whereas withering was observed in 60% flowers. At the time of excision ovary in good condition were obtained from 18 flowers only but no ovule was obtained in healthy condition, hence, could not be cultured *in vitro*.

Sixty five crosses of *T. alexandrinum* x *T. hybridum* were attempted using emasculation followed with pollination method and left for seed set in natural condition. There was no seed set.

T. alexandrinum x *T. purpureum*

Out of 47 crosses attempted in this combination, no withering of floral parts were observed and two ovules were found invariably in all the ovaries which indicated that there was no fertilization. These ovules could not be germinated under culture conditions.

In 35 crosses attempted between *T. alexandrinum* and *T. purpureum*, no seed formation was observed in natural condition. However, withering was noticed in some flowers.

DISCUSSION

5. DISCUSSION

Trifolium is a large genus comprising of species distributed in various agro-ecological zones ranging from subtropical to temperate parts of the world. Clovers are widely known and easily recognizable since ancient times even before Linnaeus who recorded about 40 species and made an attempt to group in to natural units (Zohary, 1972). *Trifolium* is one of the most difficult genus as seen by the fact that for the 238 existing species about 1100 binomials have been published (Zohary, 1972). Lojacono (1883) made first attempt to complete a kind of key to over 200 species including American species known at that time. Sporadic reports are available about affinity among species within the genus but these reports are mainly confined to the temperate species. *T. alexandrinum* is an important fodder species in India, which necessitates to understand its affinity with other species of the genus.

The present study on genus *Trifolium* is an attempt to know the affinity among various *Trifolium* species and their relationship with *T. alexandrinum*. Morphological characters, cytological studies, isozymic and protein variations and interspecific crossability were the measures to estimate the affinity among species.

5.1 Morphology

Morphological data was recorded on 25 species (represented by 125 accessions) of genus *Trifolium*. Most of the species were annual. Only two species i.e. *T. repens* and *T. hybridum* behaved as perennial in agroclimatic condition at Jhansi. The species like *T. alpestre*, *T. medium* which are reported to be perennial could not survive in the high temperature during summer and died without flowering. As such in the genus annuality and perenniality is widespread and found in 2:1 ratio (Zohary, 1972). Most of the perennial species (60 out of 90) are included in 'Lotoidea' whereas section 'Trifolium' has only 20 perennials out of 70. Annuality here, as elsewhere is derivative and more progressive at least in regard to the reproductive organization. Annuality also helped in spread of species into less mesic habitat leading to abundance of annuals in Mediterranean and scarcity in higher latitude and altitude. Thus, on the basis of perenniality / annuality 25 species of our study can be placed in two groups, the first comprising of four species (*T.*

repens, *T. hybridum*, *T. alpestre* and *T. medium*) and the second includes 21 species.

The morphological observations recorded for various accessions of different *Trifolium* species did not reveal considerable variation among different accessions except in *T. nigrescens*, *T. subterraneum*, *T. resupinatum* and *T. campestre*. The *T. subterraneum* accession (EC 401717) was marked for pink flowers and above ground seed formation, whereas, in rest of the accessions seed set was underground. One of the accessions of *T. nigrescens* (EC 425047) was marked for deep cut in leaflet margin. The leaflets of these plants were obovate / round initially but during development the furrows got deepened. Considerable intraspecies variation was marked in *T. resupinatum*. The plants of different accessions ranged from prostrate to erect in nature. Number of leaves per plant also varied to the great extent although the high number of leaves were common with prostrate accessions which possessed small leaflets and profuse branching. Variation among different collections of 'Shaftal' has been reported earlier also (Shukla & Malaviya, 1988). Fifty advance breeding lines of 'Berseem' did not show enough variation for various characters studied. 'Fahli' (IL 40010), 'Saidi' (EC 329299), diploid and tetraploid lines showed distinct morphological features. Tetraploids possessed broad leaves with serrate margins and hairy surface against entire and less hairy leaves of diploids. 'Fahli' and 'Saidi' were found to be erect with no branching from the basal region which was characteristic of all other 'Mescavi' lines. 'Fahli' and 'Saidi' lines did not show multicut nature. The morphology and cutting behavior of the three types of 'Berseem' varieties i.e. 'Fahli', 'Saidi' and 'Mescavi' have been reported by earlier workers also (Malaviya and Rao, 1997)

Certain morphological traits considered as markers for evolutionary trend were observed in different species e.g. from bracteate to ebracteate flower. In the primitive section of 'Lotoidea' and 'Mistyllus' presence of bracts was noticed whereas section *Trifolium*, except a few species, completely lacks bracts. In the present study *T. glomeratum*, *T. repens*, *T. resupinatum*, *T. spumosum*, *T. hybridum*, *T. nigrescens* and *T. retusum* possessed bracts whereas remaining species did not. The bractless state constitute the most advanced stage in the evolution of the clover

head and it is evident by the fact that the most elaborated sections of 'Trifolium' are bractless, or only rudimentarily bracteate (Zohary, 1972).

The closure of the calyx is, quite essential in germination biology and a feature of survival value. The successive degrees in the evolution of this characteristic can be clearly followed within the large section of 'Trifolium'. Here, in some perennials almost open throats are still met with, while among the annuals, there are all kinds of devices for closing the throat of the calyx. This ranges from hairy or callous rings at the inside of the throat to two-lipped callous outgrowths which shut the calyx very tightly. The type and degree of the closure is a diagnostic characteristic in the section (Zohary, 1972). According to this criteria the species like *T. glomeratum*, *T. repens*, *T. hybridum*, *T. retusum*, *T. nigrescens*, *T. subterraneum*, *T. vesiculosum* and *T. resupinatum* observed for open type of calyx throat can be grouped in one and be treated as primitive species. In the second group of species (*T. lappaceum*, *T. cherleri*, *T. pratense*, *T. arvense*, *T. angustifolium*, *T. purpureum*, *T. incarnatum*, *T. alexandrinum*, *T. apertum*, *T. echinatum*) the calyx throat was closed. This group of species comprising mainly of annual species can be considered as advanced.

Zohary (1972) observed that there is a well traceable trend in the evolution of the pod from a typical many-seeded and suturally dehiscent legume to an utricle-like, one seeded body with a non-dehiscent, irregularly breaking pericarp which is often membranous in the lower part and somewhat leathery and cup-shaped in the upper part. The processes connected with this trend are: (a) reduction of the seed number to one, (b) corresponding shortening of the pod, (c) inclusion of the pod within the calyx tube, (d) membranization of the pericarp, (e) loss of separation tissue along the sutural zones, (f) shutting the calyx throat and so converting the fruiting calyx into a diaspore. In the present study only *T. tembense* possessed 5 to 6 seed per pod followed by *T. repens* and *T. incarnatum* with 1 to 2 seeds per pod, in rest of the species single seed per pod was observed.

On the basis of various morphological traits, the genus that has been grouped in different sections and further smaller groups. According to Flora Europea (Coombe, 1968) the genus is divided into two major groups. The first, group of species were marked for staminate tube, stamens diadelphous and legume included in calyx, with five or more leaflets. In the second group, all trifoliate species have been grouped into two sub groups based on calyx venation. A few species like *T. hybridum*, *T. repens*, *T. campestre* in this study belonged to the first category with 5 to 6 veins and scarious fruit, whereas all other species of our study belonged to the second group with 10 to 20 veins in calyx and deciduous petals. This group has been further clustered into two sub-groups based on floral characters such as presence of bracts and number of seeds per legume. Thus, *T. glomeratum*, *T. repens*, *T. resupinatum*, *T. vesiculosum*, *T. spumosum*, *T. hybridum*, *T. nigrescens*, *T. retusum* belong to first sub-group with flower subtended by small / connate bracts, throat of calyx not closed by a ring of hair or by an annular or bilabiate callosity. The second sub-group incorporates species like *T. subterraneum*, *T. alpestre*, *T. medium*, *T. lappaceum*, *T. hirtum*, *T. cherleri*, *T. pratense*, *T. arvense*, *T. angustifolium*, *T. purpureum*, *T. incarnatum*, *T. alexandrinum*, *T. apertum*, and *T. echinatum* with ebracteate flower buds but heads sometimes involucreate throughout calyx closed. In Flora Europea (Coombe, 1968) the genus has been divided in subgenus 'Falcatula' (Brot.) 'Lotoidea' Pers. and 'Trifolium'. The subgenus has been divided into sections. The grouping of various species in different subgenera and sections is given in Table 2.1. From this classification based on key characters *T. alexandrinum*, *T. apertum* and *T. echinatum* formed one group which is very close to another group of *T. arvense*, *T. angustifolium*, *T. purpureum* and *T. incarnatum*. In our study also the morphological character of leaves, inflorescence and pod showed closeness among *T. alexandrinum*, *T. apertum* and *T. echinatum*.

Although wide variation for branch number, leaves per plant, petiole length, leaflet length and breadth and stipule character was observed among different species, Euclidian clustering of various accessions showed total nine clusters. The cluster comprising of all the *T. alexandrinum* accessions showed maximum distance from cluster comprising of two *T. resupinatum* accessions. The closest species to

the cluster of *T. alexandrinum* was *T. purpureum* followed with *T. hybridum*, *T. nigrescens* and three accessions of *T. resupinatum*.

5.2. Cytology

In any discussion relating to assessment of phylogeny and evolutionary status of a group, genus or species, cytogenetical data provide valuable clues. Works of Babcock (1947) on *Crepis*, Navashin (1926) and Cleland (1962) on *Oenothera*, Mather (1932) on *Crocus*, Manton (1932) on Cruciferae and Levan (1931, 1932, 1934, 1935 a,b) on *Allium* are some of the classical example in this regard. Darlington (1963) and Stebbins (1950, 1971) have discussed in detail some of the important aspects of chromosome morphology which have been found useful in understanding the evolutionary problems.

The behavior of chromosomes during meiosis throws light on their structural plan. Studies on pairing of homologues chromosomes, their configuration and chiasmata frequencies are helpful in understanding the phylogeny and evolution of a species. Darlington (1939) has pointed out that the amount of crossing over in each chromosome is determined by chiasmata frequency. He defined the recombination index as 'Sum of the haploid number of chromosomes and of the average chiasmata frequency of all the chromosomes in a meiotic cell'. So it is obvious that higher the recombination index, the greater will be the chance of gene recombinations. The new gene combinations may be obtained in any generation by which chances of natural selection increases. The processes of natural selection is restricted in the plants with low recombination indices. The low chromosome number and their small size are important factors in not allowing chiasmata frequencies to be high and hence low recombination index. Darlington (1957) and Stebbins (1950, 1971) have discussed at length how recombination index is related with breeding system of a species. Inbreeders have usually a high recombination index while the out breeders have a low one. A low recombination index greatly increases genetic linkages which seem to provide genetic constancy to the strictly out breeding individuals.

As regards somatic chromosomes compliment among the *Trifolium* species under study, *T. alexandrinum*, *T. constantinopolitanum*, *T. hirtum*, *T. vesiculosum*,

T. resupinatum and *T. hybridum* possessed $2n=16$ chromosomes whereas *T. incarnatum* and *T. campestre* possessed $2n=14$ and *T. cherleri* $2n=10$ chromosomes. Somatic chromosome number was in confirmation with earlier reports. Meiosis in all the species was near normal and regular bivalent formation was observed except a few PMCs observed with univalent/ quadrivalent/ trivalent formation. The occurrence of univalents indicates the non homology between certain chromosomes in the complement. Chromosome may fail to pair either because they are non homologous or because the linearity of the genes in them is altered by translocation / inversion, so at pachytene homologous chromosome do not lie side by side. In both cases, they may be taken to indicate different or altered chromosome structure. It is also probable that gene mutations are responsible for failure of pairing between homologous chromosome. Thus, these species can be considered to be cytologically stable as evident from their chromosomal behavior during meiosis. On the basis of somatic chromosomal number, the species with $x=8$ can be considered to be closer to *T. alexandrinum* as compared to those which possessed $x=7$ or $x=5$.

5.3. Protein analysis

In some genera a good correspondence has been obtained between their banding pattern and their morphotaxonomic distances. Rommann *et al.* (1971) investigated total soluble protein and percent fraction protein of birdsfoot trefoil (*Lotus corniculatus* L.), White clover (*T. repens* L.) and four varieties of alfalfa (*Medicago sativa* L.) using polyacrylamide gel electrophoresis. They could distinguish between the different taxa by their electrophoretic patterns. Ladizinsky (1979) also found that seed protein electrophoresis profiles of the cultivated lentil (*Lens culinaris* Medicus), the wild species *L. orientalis* Medicus and *L. nigricans* Medicus were similar, corroborating the close morphological and cytogenetic affinities of these three taxa. Similarly, Orf *et al.* (1980) analyzed soybean seeds from 3338 accessions and confirmed that seed protein electrophoresis was a useful technique for classifying soybean cultivars into broad categories. Przybylska *et al.* (1979) analyzed reduced legumin and vicilin fractions from 21 distant related *Pisum* lines by SDS - polyacrylamide gel electrophoresis (PAGE). They showed that electrophoretic data indicated distinctness of *P. fulvum* and *P. abyssinicum*. Matta *et*

al. (1981) showed a wide range of heterogeneity in the subunits of Legumin isolated from seeds of *Vicia faba* L. by SDS - polyacrlimide gel electrophoresis.

On the basis of similarity index the *T. alexandrinum* lines showed maximum affinity with *T. apertum* (70-87%) followed with *T. purpureum* (72%) and *T. vesiculosum* (56-62%). Fifty four per cent similarity of *T. alexandrinum* was also observed with *T. hirtum*. The *T. alexandrinum* accessions showed maximum distance from *T. pratense* (26 to 33% similarity) followed with *T. hybridum* (30% similarity) and 37 -46% with *T. echinatum*. Thus, *T. alexandrinum* can be said to have closest affinity with *T. apertum* followed with *T. purpureum*. It is also to note that *T. alexandrinum* has 11 out of 12 bands, common with *T. apertum* and 9 bands common with *T. purpureum*, although *T. purpureum* and *T. apertum* both had some additional bands and some being absent. The next close species to this group of three species (*T. alexandrinum*, *T. apertum* and *T. purpureum*) was *T. vesiculosum* showing 57% similarity with this cluster. This group of 4 species was 52% similar with another group of three species comprising of *T. resupinatum*, *T. repens* and *T. lappaceum*. *T. angustifolium*, *T. pratense*, *T. echinatum*, *T. hybridum* and *T. hirtum* formed small group of 1 to 2 species and were placed quite away from the cluster of *T. alexandrinum*.

Morphological characteristics of *T. apertum* (which closely resembled *T. alexandrinum*) and its crossability with *T. alexandrinum* (using embryo rescue) supports the finding of leaf protein analysis that the two species have close affinity.

Abdel-Tawab et.al. 1987 on the basis of seed protein electrophoretic profile, placed *T. alexandrinum* ('Miskawi') in a separate group whereas *T. alexandrinum* ('Fahli') was placed with *T. lappaceum*, *T. pratense*, *T. medium* and *T. hirtum*. However, on the basis of SDS PAGE analysis of seed protein they have found that the two cultivars of *T.alexandrinum*. 'Miskawi' and 'Fahli' were similar in all bands. Besides, their banding patterns were similar to those of *T. pratense* and *T. medium* except band 3 which was absent in the cultivars of *T. alexandrinum*. The two species, *T. pratense* and *T. medium* showed identical SDS PAGE patterns which indicate their close phylogenetic relationships. Such close relationship was reported by Stepler (1965), Abdel-Tawab (1970) and Selim et al. (1977). Abdel-Tawab

(1970) reported that *T. lappaceum* gave patterns which were dissimilar with rest of the species involved. The same was true for *T. hirtum*. *T. subterraneum* was similar to *T. fragiferum* in all bands except band 11, which was unique for *T. subterraneum*. The pattern of *T. resupinatum* was identical to that of *T. hybridum* even though they belong to different subgenera. This was in disagreement with the proposals of Starzycki (1961, 1959). *T. nigrescens* gave an SDS PAGE pattern which was closer to that of *T. hybridum* than to that of *T. repens*.

The protein content estimated in different accessions of *T. alexandrinum* ranged from 18.88 to 21.32% in diploid and from 19.81 to 22.52 in tetraploid lines. These tetraploid lines showed better crude protein content than the variety 'Wardan'. The range of protein content observed in this study falls within the range as reported earlier in 'Berseem' (Mahanta *et al.*, 1999). The protein content on dry matter basis in 'Berseem' has been found to be more or less the same range for first cut to the last cut (Guessous, 1981).

5.4. Isozyme Study

Isozyme analysis has been used widely to estimate genetic variability of populations. This method has been useful in addressing questions on genetic structure of population and their conservation (Brown, 1978). Knowledge of the genetic diversity of species is particularly important, since modern breeding practices involving selections for high biomass have narrowed the genetic diversity of cultivated crops. This reduction in genetic diversity could severely limit future breeding programs for adaptive traits such as resistance to biotic stresses and reduced stability of crop yields. Isozyme markers are more reliable than the morphological data as they are least affected by the environmental factors.

Oram *et al.* (1987) surveyed isozymes coding for 27 loci for a set of 20 accessions of *Cicer arietinum* of diverse geographic origins and found that only four loci were polymorphic. Tuwafe *et al.* (1988) studied isozyme variability with a large number of cultivated chickpea germplasm accessions from 25 countries and found only four polymorphic isozyme loci among six enzyme systems assayed. Gaur and Slinkard (1990 a & b, 1991) did not find genetic variation in *Cicer arietinum*.

However, 28 isozyme loci were polymorphic in *Cicer reticulatum*. Kazan & Muehlbauer (1991) found polymorphic isozyme loci among 30 isozymes assayed in 95 accessions of *C. arietinum*, eight annual and one perennial wild species of *Cicer*.

It is evident from the isozymic pattern from different enzymes that the 65 accessions of *T. alexandrinum* possessed very little variability. In most of the cases the similarity was observed to be 95-100% except the accession number EC 329299, (a 'Saidi' type) line which was 79% similar to rest of accessions. There was no marked difference for banding pattern among the *T. alexandrinum* lines, representing various climatic conditions, except for one or two bands of peroxidase, one band each of SOD and ACP and two bands of GOT. *T. alexandrinum* lines representing various exotic lines, some collections each from Rajasthan, Punjab, Haryana, tetraploid lines, pentafoliate and red flowered plants of *T. alexandrinum* possessed 95-100% similarity, although, the red flowered and pentafoliate plants clustered at the end of the dendrogram.

In *T. alexandrinum*, 65 lines were analysed which formed 3 major groups which were again subdivided into 13 clusters. Group I comprising of single accession (EC 329299) was different ecotype ('Saidi') and has wide difference for morphological features and regeneration potential. Group II comprised of 50 lines which were grouped into cluster 2 to 9. This group showed 93% within group similarity. Many of the accessions were 100% similar indicating thereby presence of duplicates. Group III comprised of 14 accessions grouped into clusters 10 to 13. This group showed 92% within group similarity was 89.8% similar with group II. The 'Saidi' type accession showed 79.2% similarity with 'Mescavi' type accessions represented by 64 accessions grouped in cluster 2 to 13.

These berseem accessions were 75% similar with *T. constantinopolitanum* at one end and *T. alexandrinum* together with *T. constantinopolitanum* formed one cluster which was 70% similar with *T. tembense*. Thus, these two species i.e. *T. tembense* and *T. constantinopolitanum* seemed to be closest to *T. alexandrinum*. The other few species which showed close affinity with *T. alexandrinum* were *T. medium*, *T. glomeratum*, *T. apertum* and *T. echinatum* in the order of similarity. Species like *T. lappaceum*, *T. diffusum*, *T. campestre*, *T. incarnatum* and *T. argutum*

were clustered at one extreme of the dendrogram and showed only 44.8% similarity with other *Trifolium* species. If we look in to the origin and domestication of these species (Table 2.2) all these five species have domesticated in Europe and USA i.e. western hemisphere. Except for *T. incarnatum* from this group rest of the species showed less number of isozyme bands represented in them. Some of widely cultivated species in India like *T. alexandrinum* and *T. resupinatum* possessed 28 and 27 bands respectively out of total 66 bands identified but these five species showed presence of 11 to 17 bands only. Shahi *et al.* 1969 considered that with the ongoing process of domestication of species, some bands get fixed while other get eliminated but in our study the two widely cultivated (*T. alexandrinum* and *T. resupinatum*) and domesticated for more then 100 years in India show higher number of bands than European and Western species.

Based on pooled zymogram pattern *T. alexandrinum* showed maximum affinity with *T. resupinatum* (72%) similarity followed with *T. apertum* (71%). *T. purpureum* and *T. subterraneum* also showed 69% and 68% similarity with *T. alexandrinum*. *T. campestre* and *T. lappaceum* were most distantly related with *T. alexandrinum*. *T. constantinopolitanum* which showed some crossability (using embryo rescue) with *T. alexandrinum* showed closer affinity with *T. lappaceum* (72%), *T. tembense* (71%), *T. apertum* (71%) and *T. echinatum* (71%) as compared to 63% similarity with *T. alexandrinum*. The higher degree of crossability of *T. alexandrinum* with *T. apertum* but closer banding pattern similarity of *T. apertum* with *T. constantinopolitanum* leads to think that *T. apertum* might be a bridge species of the cross between *T. alexandrinum* x *T. constantinopolitanum*. The semi erect habit of *T. apertum* also looks to be intermediate nature of plant type of erect *T. alexandrinum* and prostrate *T. constantinopolitanum*.

The maximum affinity (79%) between any two species was observed between *T. spumosum* with *T. cherleri*. From the similarity index table it is clear that the species closer to (more than 60% similar) *T. alexandrinum* are *T. resupinatum* (72%) *T. apertum* (71%), *T. purpureum* (69%), *T. subterraneum* (68%), *T. nigrescens* (65%), *T. cherleri* (64%), *T. argutum* (64%) and *T. spumosum* (63%). The species which showed close affinity with the two closest species of *T.*

alexandrinum i.e. *T. resupinatum* and *T. apertum* were also identified based on similarity index. The closest species to *T. resupinatum* was *T. alexandrinum* itself followed with *T. medium* (67% similarity) whereas the closest species to *T. apertum* was *T. tembense* (73%) followed with *T. alexandrinum*, *T. alpestre* and *T. constantinopolitanum* (71% similar). Thus, there is possibility of *T. alexandrinum* and *T. resupinatum* commonly evolving from *T. medium* at one hand and *T. alexandrinum* alone evolving from *T. constantinopolitanum* through *T. apertum*.

Wide interspecific genetic diversity was observed among *Trifolium* species. *T. diffusum* was most distantly placed species out of 25 studied and showed only 40.5% similarity with other 24 species. *T. repens* also fell at the other end of dendrogram and was only 46.1% similar with other 23 species. Moderate affinity (>70 %) was observed between *T. cherleri* and *T. spumosum* (78.8%), *T. alpestre* and *T. hybridum* (77.4%), *T. alexandrinum* and *T. resupinatum* (72.4%), *T. glomeratum* and *T. apertum* (72.7%) and that between *T. lappaceum* and *T. constantinopolitanum* (72%).

This is the first attempt to study the isozymic variation for five enzymes involving 25 species represented by a total of 134 accessions. The migration zones identified for different enzyme were quite similar across the genus and hence, the band were considered only as the presence and absence of single band. Polignano *et al.*, (1998) also considered only the presence and absence of single band of malic enzyme since no reference in *Vicia faba* was available. In the present study also the presence of different bands in different accessions at each migrating zone was separately analyzed particularly in *T. alexandrinum* represented by 65 accessions, *T. resupinatum* by 12 accessions, and *T. repens* by 9 accessions but in none of the case for any enzyme for any migrating zone the frequency of presence of bands was matching to any Mendelian system suited to monomeric dimeric or tetrameric enzyme. Thus, it was considered that each band is an independent monomeric allele.

5.5. Interspecific Hybridization

The major genes for wide scale adaptability to varying climatic conditions, disease resistance, are widely distributed in several wild species of *Trifolium* which

could be incorporated into the present day cultivars provided that problem in interspecific crossability is overcome.

Compatibility between different species is an indication of close affinity between the two species. In large number of crops like *Solanum*, *Avena*, *Triticum* and *Oryza*, phylogenetic relationship has been well traced using information on interspecific compatibility relationships. In case of *Trifolium* the incompatibility barriers are very strong and natural interspecific hybridization has probably played insignificant role in speciation. Evans (1962b) reported the existence of a high degree of incompatibility between species of *Trifolium*. Considerable success in overcoming self incompatibility by relatively high temperature has been reported in *T. pratense* L. (Leffel, 1963; Kendal & Taylor, 1969.) and in *T. hybridum* L. (Townsend, 1968). Dadson (1969) has also reported that heat treatments increased the degree of interspecific compatibility among certain species of *Trifolium*. Evans (1962b) investigated that pollen tube growth was lower in the interspecific crosses but no failure of pollen germination was observed in *Trifolium*. In a genus of 237 species phylogenetic relationship, based on interspecific hybridization, have been established only among few species. In our study the closeness between the two species was considered in following order of affinity :

- (1) No response of pollination and no withering of corolla observed.
- (2) One of the ovules degenerated, petals withered.
- (3) Embryo development took place and successfully germinated.

This study indicated that there was no response of pollinating *T. alexandrinum* with *T. incarnatum* and negligible response with *T. vesiculosum* and *T. repens*. Thus, these species showed very little or negligible affinity with *T. alexandrinum*. Successful embryo germination in the *T. alexandrinum* cross with *T. apertum*, *T. constantinopolitanum*, *T. echinatum* and *T. resupinatum* shows that these four species have closer affinity with *T. alexandrinum* than other species. In a study by Trimble & Hovin (1960) and Evans (1962a) *T. repens* has shown some crossability with *T. alexandrinum*. In a study of Malaviya *et al.* (unpublished) it has been observed that in the normal course there are two ovules in *T. alexandrinum* and after fertilization one ovule gets degenerated and the other develops. The similar

phenomenon was observed in *T. alexandrinum* x *T. apertum* cross wherein development of only one ovule was observed in the withered flowers, whereas both the ovule remained healthy for a very long period where no fertilization occurred. In other crosses involving *T. constantinopolitanum*, *T. echinatum*, *T. pratense* *T. resupinatum* 0.7, 0.3, 0.11 and 0.22 ovule per ovary were observed respectively. If this is considered as criteria for measuring affinity of species *T. constantinopolitanum* and *T. apertum* seemed to be the closest species to *T. alexandrinum* followed with *T. resupinatum* and *T. echinatum*.

5.6. Phylogeny

One hundred fifty species of this genus (belonging to seven sections out of total 8 sections) are represented in Mediterranean region. Among these, six sections have their predominant number of species in this zone. Section 'Trifolium' has its center of differentiation in East Mediterranean. On the basis of distribution of different species in various region Meusel *et al.* (1965) and Bobrov (1947, 1967) tried to find out the linkages among different sections and also the species of *Trifolium*. Zohary (1972)) considered the fact that section 'Lotoidea' is the most primitive and widely spread section and has played important role in evolution of the genus. Looking at the genus from the stand point of variation and polymorphism Zohary (1972) considered the possibility of an ancestral heterophyletic origin of the genus, yet he discussed the phylogeny of the genus considering it as a discrete taxonomic entity. 'Lotoidea' with its sub section 'Lupinaster' is considered as an intergeneric link and sub section 'Neolagopus' as a link joining 'Lotoidea' with other sections primarily with section 'Trifolium'. Bobrov (1947 & 1967) recommended the separation of 'Lotoidea' from the genus based on his observation of morphological similarity of the section with 'Trifoliatrum' but Zohary (1972) opined that the section should remain as it is.

From the distribution layout of the genus it is obvious that within the temperate latitude of the Northern hemisphere, NW America constitute the largest section of 'Trifolium' (about 50 species mainly of Section 'Lotoidea') while the eastern states of America almost lack representatives of this genus. Diversity of forms crowded in NW America, absence of American representative in Eurasian

'Trifolium' sections and the possible migratory root of other taxa leads to think that possibly the species like *T. pacificum* found in Far East must have migrated across the pacific way i.e. NE America to E Asia. This was possibly the first step in the tremendous process of evolution in 'Trifolium' that took place in E Hemisphere. It was a swarm of species of white clover group or one close to each that gave rise the formation of other sub section of 'Lotoidea' and six other sections of Trifolium on Eurasian and African ground. The further differentiation of these species took place in E Hemisphere during long span of Neogene period. Further migration of the species from Mediterranean to Tropical East Africa took place through Afro-alpine-Mediterranean line. Only two sub sections of i.e. 'Ochreata' and 'Loxospermum' represented in Africa evolved from the old member of sub section 'Amoria'.

Wexelsen (1928) suggested that hybridization played a minor role in evolution of *Trifolium* because they were rarely found in nature and not easily obtained experimentally. Evolutionary trend in 'Trifolium' section of the genus are towards a reduction of basic chromosome number (Pritchard, 1969). It is possible that progenitor species of high chromosome number will show a closer relationship to a species of lower chromosome number.

There appears to have been two evolutionary trends in the genus. Polyploidy has occurred to a marked extent in one of the more primitive subgenera, which has an extensive distribution in Africa, America, and Europe. In the more specialized and evolutionary advanced subgenera polyploidy is much less common and the main trend has been a reduction of the basic chromosome number. This reduction has occurred principally among the species which are concentrated around the eastern parts of the Mediterranean region (Pritchard, 1969).

5.7. Conclusion

Most of the earlier studies about phylogeny and classification of the genus were based on morphological studies and geographical distribution. Temperate species were also compared phylogenetically on the basis of interspecific hybridization and cytology. However, the present study on genetic similarity at

inter and intra specific level among different accessions belonging to 26 species of the genus is an attempt to get a better insight into the relatedness of different species.

Our study aimed mainly to work out genetic diversity in *T. alexandrinum* and its relatedness with other species of *Trifolium*. The study confirmed the earlier observations that the genetic base of berseem is quite narrow. Out of 65 accessions studied by isozymic method more than 95% similarity was observed among 64 accessions. The only accession (EC 329299), a 'Saidi' ecotype was different.

Earlier reports indicated similarity among *T. alexandrinum*, *T. apertum*, *T. constantinopolitanum*. Our study has also shown high genetic similarity based on isozyme analysis, supported by cytological and crossability studies that these three species as well as *T. echinatum* and *T. resupinatum* are closely related. Successful in vitro germination of interspecific embryos also confirm high degree of affinity.

T. alexandrinum was found to be closer to another cultivated clover (*T. resupinatum*) in sub-tropical part of India. Morphological data analysis show wide diversity among the germplasm of *T. resupinatum*. Two accessions of *T. resupinatum* were found to have some closeness with *T. alexandrinum* group (low intercluster distance 2.830). Isozymic study show moderate similarity between these two species (72%). Cytological and crossability study also confirm this similarity and in vitro embryo germination could successfully be obtained in this cross. Selim *et al.* (1977) have also obtained successful hybrids in these cross combinations.

Isozymic study was found to be more reliable than the morphological observations. Clustering of 134 accessions belonging to 25 different species showed no overlapping of accessions. All the accessions of any particular species were placed together in the dendrogram, thereby confirming the high efficacy of this analysis.

SUMMARY

6. SUMMARY

The genus *Trifolium* commonly called clovers comprises of 237 annual and perennial species, out of which a few are agriculturally important as cultivated and pasture crops. The important perennial pasture clover *T. repens* (white clover), *T. hybridum* (alsike clover), *T. pratense* (red clover) and *T. ambiguum* (Caucasian clover) are widely distributed in the temperate and sub-temperate regions of the world. The annual types *T. resupinatum* (Persian clover) and *T. alexandrinum* (Egyptian clover or berseem) are commonly cultivated as winter annuals in the tropical and subtropical regions.

Berseem (*T. alexandrinum*) is the most important winter season annual fodder legume and is cultivated in an area of around two million hectares in India. The merit of this crop lies in its multicut nature (4-8 cuts), long duration of green fodder availability (November to April) and very high yield (85 t/ha), good quality (20% crude protein), high digestibility (up to 65%), palatability and high N₂-fixing ability.

Considering its high production potential and wide adaptation in the tropical and sub tropical zones of the country, the crop has attracted serious attention for its further genetic improvement, but no major breakthrough was achieved in the last two decades. The main bottleneck is the narrow genetic base of the crop which is due to the fact that the crop is not native to India, where, it was introduced in 1904 and one of its cultivar or ecotype 'Mescavi' or 'Miskawi' became adapted. Most of the present day cultivars are derivatives of this ecotype. Selection for high biomass has led to erosion of its genetic diversity and the present germplasm maintained at various universities / research organizations lack the genetic diversity.

Introgression of desired traits through intervarietal and interspecific hybridization seems to be only plausible answer to broaden the genetic base. The work in this direction is hampered because of failure of interspecific crosses in natural conditions. The genes for desirable traits such as disease resistance and wide scale adaptability to varying soil/climatic conditions are reported to be widely distributed in several wild/allied species. These genes could be incorporated into the

present day cultivars of the most commonly grown species in India *Trifolium alexandrinum* provided the affinity with other wild/temperate/sub-temperate species of the genus is clear. Very little work has been done on affinity of various species, within the genus *Trifolium*. Although sporadic reports are available about affinity of temperate and sub-temperate species, reports on their relationship with *T. alexandrinum* or with other tropical species is lacking.

In light of the above, the present investigation entitled "Studies on inter and intra genetic distance among *Trifolium* species based on morphological, biochemical and cytogenetical attributes" has been envisaged with following objectives:

- 1) To determine phenotypic variations within and between different *Trifolium* species and their biochemical characterization.
- 2) To work out interspecific compatibility relationships among different species and to establish their relative affinity.
- 3) To estimate genetic similarity among various species.

Accessions of different *Trifolium* sp. were procured through various sources. Indigenous *Trifolium* sp. were procured from different research Institutions/ Universities of India including IGFRI, Jhansi. Many advanced breeding lines and natural as well as induced mutants / variants, exotic species procured through NBPGR were taken from *Trifolium* improvement project of IGFRI. The details of various species and number of accessions used in the present study are as under:

T. apertum (1), *T. resupinatum* (12), *T. glomeratum* (3), *T. subterraneum* (5), *T. vesiculosum* (2), *T. hybridum* (5), *T. repens* (9), *T. pratense* (9), *T. incarnatum* (2), *T. hirtum* (4), *T. diffusum* (1), *T. spumosum* (1), *T. lappaceum* (1), *T. argutum* (1), *T. arvense* (1), *T. campestre* (4), *T. constantinopolitanum* (1), *T. alexandrinum* (69), *T. cherleri* (1), *T. nigrescens* (3), *T. echinatum* (5), *T. medium* (1), *T. alpestre* (2), *T. tembenense* (4), *T. purpureum* (2), *T. angustifolium* (2), *T. retusum* (1)

To get a comprehensive picture of genetic similarity, certain parameters were used in a systematic way. All the accessions were subjected to detailed morphological and isozymic studies. The data were analysed and genomic similarity was worked out between accessions and species using appropriate computer

software. These studies were followed by more careful examination of selected genotypes /species using other parameters such as cytological examination, crude protein estimation, leaf protein qualitative analysis and interspecific hybridisation. All these factors were taken into consideration to arrive at any inference.

The plants of different accessions of *Trifolium* were raised in nursery and Central Research farm of IGFR, Jhansi for two consecutive years in 1998 and 1999. Morphological observations were recorded on various qualitative as well as quantitative traits. Meiotic studies were carried out in pollen mother cells (PMCs) from young flower buds. *T. alexandrinum* and other *Trifolium* species were compared for the five enzymes- Peroxidase, Esterase, Superoxide dismutase (SOD), Acid phosphatase (ACP) and Glutamate oxalo acetate transaminase (GOT) using Horizontal starch gel electrophoresis technique. Protein estimation was done both quantitatively and qualitatively. Crude protein per cent was estimated by conventional Nitrogen estimation method and leaf protein analysis was done using PAGE (Polyacrylamide vertical gel electrophoresis) system.

Numerical data recorded for various morphological traits were analyzed statistically. Genetic similarity between different accessions of any particular species and among different species were worked out using non-hierarchical Euclidian cluster analysis for grouping of genotypes. A binary data matrix reflecting the presence or absence of specific isozyme band was generated for all the accessions of *Trifolium* species. The genetic similarities (GS) between line i and j were estimated using the formula of Dice (1945). Dendrogram was generated using the unweighted-pair-group method average (UPGMA) clustering procedure using computer software NTSYS-PC version 1.60. Interspecific crosses were attempted using emasculation followed with pollination method to find the genetic compatibility of the species with *T. alexandrinum*.

6.1. Morphological studies

Morphological observation were recorded on 125 accessions representing 25 species of *Trifolium*. Wide diversity for various morphological characters in 25 *Trifolium* species was noticed. Considerable variation for branch number was observed among different *Trifolium* species. Number of primary branches per plant

ranged from 1 to 18.3. Maximum 18.3 branches per plant was recorded in *T. nigrescens* followed with 14.2 branches each in *T. hirtum* and *T. resupinatum*. Single branch was noticed in *T. medium* followed with 3 branches in *T. tembense* and 3.1 per plant in *T. pratense*. Most of the species possessed 5 to 8 branches per plant.

Leafiness is an important forage trait and high degree of variation for this trait was recorded among different species. Maximum 309.6 leaves were present in prostrate line of *T. resupinatum* followed with 259 in *T. echinatum*. Minimum number of leaves i.e. 7.7 were observed in *T. medium* followed with 16.2 in *T. pratense*.

Petioles of *T. incarnatum*, *T. resupinatum*, *T. arvense*, *T. constantinopolitanum*, *T. apertum*, *T. subterraneum* were observed to be quite long and ranged from 7 to 8.4 cm whereas petioles of *T. echinatum*, *T. campestre*, *T. angustifolium*, *T. tembense* were quite short ranging from 1.0 to 2.0 cm. *T. alexandrinum*, *T. hirtum*, *T. argutum*, *T. pratense*, *T. diffusum*, *T. glomeratum* and *T. hybridum* possessed medium sized petioles ranging from 5.0 to 6.4 cm.

Leaflets of *T. alexandrinum* were longest (3.7 cm) followed by that of *T. purpureum* (3.1 cm). *T. lappaceum*, *T. cherleri*, *T. hirtum*, *T. diffusum*, *T. spumosum*, *T. argutum*, *T. arvense*, *T. campestre*, *T. tembense*, *T. apertum*, *T. constantinopolitanum*, *T. repens*, *T. subterraneum*, *T. glomeratum*, *T. incarnatum*, *T. alpestre* and *T. echinatum* possessed small leaflets ranging from 0.8 to 1.8 cm. Leaflets of *T. incarnatum* were widest (1.6 cm) followed with that in three species viz. *T. alexandrinum*, *T. pratense*, *T. resupinatum* (1.5 cm) whereas minimum leaf breadth was noticed in *T. angustifolium* (0.4 cm). The species viz. *T. lappaceum*, *T. campestre*, *T. cherleri*, *T. argutum*, *T. echinatum*, *T. alpestre*, *T. purpureum*, *T. constantinopolitanum*, *T. hirtum*, *T. repens*, *T. glomeratum*, *T. medium*, *T. apertum*, *T. arvense*, *T. diffusum*, *T. spumosum* and *T. hybridum* possessed medium broad leaves (0.7 to 1.3 cm).

The fused portion of stipules in *T. purpureum* was longest (2.1 cm) followed with that of *T. alexandrinum* (1.8 cm). The fused portion of stipules in *T. campestre*, *T. argutum*, *T. incarnatum*, *T. tembense*, *T. repens*, *T. glomeratum*, *T. cherleri*, and

T. medium was quite short ranging from 0.4 to 0.6 cm. Free portion of stipules in *T. purpureum*, *T. hybridum*, *T. alexandrinum* and *T. resupinatum* was observed to be quite long and ranged from 1.1 to 1.5 cm whereas the stipule of *T. lappaceum*, *T. campestre*, *T. incarnatum*, *T. cherleri* and *T. tembense* were quite short (± 0.4 cm). The longest total stipule length was observed in *T. purpureum* (3.6 cm) followed with 2.9 cm long stipules in *T. alexandrinum*. Small stipules ranging from 0.8 to 1.1 cm were observed in *T. campestre*, *T. tembense*, *T. argutum*, *T. glomeratum*, *T. incarnatum*, *T. cherleri*, *T. repens* and *T. medium*.

6.1.1. Clustering of accessions of *Trifolium* species based on morphology

Clustering of seventy five accessions belonging to 25 *Trifolium* species (excluding fifty advanced breeding lines of *T. alexandrinum*) based on 8 morphological traits revealed a total of nine clusters. Maximum 22 accessions were present in cluster number 5 followed with seventeen accessions in cluster number 1. Cluster number 4 and 8 were represented with 2 accessions each. The maximum distance was observed between cluster number 8 and 9 (i.e. 7.46) followed with intercluster distance of cluster 8 with clusters 1 and 2. Cluster number 9 and 1 showed least distance of 1.74 followed with 1.99 between cluster 5 and 1. All nine *T. alexandrinum* accessions formed a separate cluster whereas in cluster number 4, two accessions of *T. purpureum* EC 425069 and EC 425070 were present.

Wide intra-species variation was recorded in *T. resupinatum* accessions. Out of 5 accessions of *T. resupinatum* three accessions (SH 98-73, SH 98-86 and SH 98-15) were present along with one accession each of *T. hybridum* (EC 425029) and *T. nigrescens* (EC 425047) in cluster number 6. Remaining two accessions of *T. resupinatum* (SH 98-36 and SH 98-72) made a separate cluster number 8.

Similarly, five accessions of *T. hybridum* showed wide variation among themselves. Two accessions (EC 401702 and EC 401701) were grouped with 3 accessions of *T. repens* (EC 401708, EC 400985, EC 400986) and one accession of *T. glomeratum* (EC 402170) in cluster number 1 whereas two accessions of *T. hybridum* (EC 425030 and EC 425032) were present in cluster number 5 with *T. incarnatum* (EC 402164, IG 96-111) and three accessions of *T. glomeratum* (EC 401700, EC 402170, EC 425033). *T. hybridum* (EC 425029) was present in cluster

number 6 with one accession of *T. nigrescens* (EC 425047) and three accession of *T. resupinatum* (SH 98-73, SH 98-86 and SH 98-15). Nine accessions of *T. pratense* were present in two cluster i.e. in cluster number 5 and 9. In cluster number 9 only 2 accessions of *T. pratense* (EC 401721 and EC 401720) were present with *T. medium* (EC 425045), *T. lembense* (EC 425064, EC 425066, EC 425065) and *T. campestre* (EC 425027).

Cluster number 2 comprising of 9 accessions of *T. alexandrinum* showed the maximum distance (6.199) with cluster number 8 in which only two accessions of *T. resupinatum* (SH 98-36 and SH 98-72) were present whereas it showed minimum distance (2.634) from cluster number 4 comprising of 2 accessions of *T. purpureum* (EC 425069 and EC 425070).

Thus, wide variation for branch number, leaves per plant, petiole length, leaflet length and breadth and stipule character was observed among different species. *T. alexandrinum* accessions showed maximum distance from *T. resupinatum* accessions. The closest species to the cluster of *T. alexandrinum* was *T. purpureum* followed with *T. hybridum*, *T. nigrescens* and three accessions of *T. resupinatum*. The study indicated that within species wide genetic diversity is present among various accessions. Thus, while attempting gene transfer through interspecific hybridization importance should be given to use specific genotypes, which show close affinity.

Morphological characters like closure of calyx indicate the evolutionary trend and affinity among different species of the genus. In some perennials almost open throats are still met with, while among the annuals, there are all kinds of devices for closing the throat of the calyx. This ranges from hairy or callous rings at the inside of the throat to two- lipped callous outgrowths which shut the calyx very tightly. According to this criteria the species like *T. glomeratum*, *T. repens*, *T. hybridum*, *T. retusum*, *T. nigrescens*, *T. subterraneum*, *T. vesiculosum* and *T. resupinatum* observed for open type of calyx throat can be grouped in one and be treated as primitive species. In the second group (*T. alpestre*, *T. medium*, *T. cherleri*, *T. lappaceum*, *T. pratense*, *T. angustifolium*, *T. purpureum*, *T. incarnatum*, *T.*

alexandrinum, *T. apertum*, *T. echinatum*) the calyx throat was closed and the legume possessed one to two seeds. This group of species comprising mainly of annual species can be considered as advanced.

6.1.2. Clustering of *T. alexandrinum* lines based on morphology

Clustering of fifty *T. alexandrinum* lines based on 16 characters was done using Euclidian cluster analysis method. A total of 8 clusters were observed. Cluster number 1 and 6 were observed with maximum number of eight accessions followed with 7 accessions each in cluster number 3 and 7. Cluster number 2 and 8 were with least number of accessions i.e. 4 accessions each. Maximum distance was observed between cluster number 8 and 5 (6.57) followed with 6.26 inter-cluster distance between cluster number 3 and 2. Cluster number 1 showed least distance with cluster number 6 i.e. 2.526 followed with 3.190 distance noticed between cluster number 6 and 7. Both the clusters 1 and 6 were found closer and with same number of accessions i.e. 8 accessions. Cluster number 1 was represented with two JHB accessions, one each from Rajasthan, Haryana and Punjab and three 'Wardan' lines whereas in cluster number 6, five JHB accessions, two Rajasthan accessions and one exotic accession (IL 40014) were present.

6.2. Cytological studies

As regards somatic chromosomes complement among the *Trifolium*, species under study, *T. alexandrinum*, *T. constantinopolitanum*, *T. hirtum*, *T. vesiculosum*, *T. resupinatum* and *T. hybridum* possessed $2n=16$ chromosomes whereas *T. incarnatum* and *T. campestre* possessed $2n=14$ and *T. cherleri* $2n=10$ chromosomes. Somatic chromosome number was in confirmation with earlier reports. Meiosis in all the species was near normal and regular bivalent formation was observed except in a few PMCs observed with univalent/ quadrivalent/trivalent formation. The occurrence of univalents indicates the non homology between certain chromosomes in the complement. Chromosome may fail to pair either because they are non homologous or because the linearity of the genes in them is altered by translocation / inversion, so at pachytene homologous chromosome do not lie side by side. In both cases, they may be taken to indicate different or altered chromosome structure. It is also probable that gene mutations are responsible for failure of pairing between

homologous chromosomes. Thus, these species can be considered to be cytologically stable as evident from their chromosomal behavior during meiosis. On the basis of somatic chromosomal number, the species with $x=8$ can be considered to be closer to *T. alexandrinum* as compared to those which possessed $x=7$ or $x=5$.

6.3. Isozyme studies

Isozyme markers are less subjected to environmental factors as compared to morphological parameters and hence are more reliable. Isozymes are product of direct gene action and hence can be visualized as a potent tool of estimating genetic similarity.

One hundred thirty four accessions belonging to 25 different species of genus *Trifolium* were subjected to horizontal starch gel electrophoresis using discontinuous buffer system. The bands were scored and numbered on the basis of their relative mobility towards anodal / cathodal ends.

6.3.1. Banding Pattern of different enzymes

The study for esterase isozymes showed presence of six distinct migration zones with a total of 18 bands. Slowest zone was with single band whereas fastest zone was most polymorphic represented by five bands. Two bands each in second and fifth migration zone and four each in third and fourth migration zones were identified. Relative mobility (RM) of band ranged from 0.12 to 0.96 RM.

The study for SOD isozymes showed presence of eight bands distributed through three distinct migration zones. There were two bands each in first and second zone whereas four bands were identified in the third zone. The relative mobility (RM) varied from 0.49 to 0.9 RM.

GOT isozyme banding pattern in *Trifolium* revealed the presence of three migration zones and a total of ten bands distributed through these three zones. First zone was represented by three bands, second by five bands and the third by two bands. The relative mobility (RM) of bands ranged from 0.11 to 0.65.

The study for ACP isozymes revealed the presence of eight bands in three distinct migration zones. In the slowest zone one band was present whereas five and two bands were noticed in second and third migration zones respectively. The slowest band was observed at 0.45 RM and the fastest at 0.88 RM.

Eleven bands were present in eight peroxidase migration zones on either side. Towards anodal side five migration zones were identified. Zone 1, 4 and 5 were represented by single band whereas 3 and 5 bands were present in zone two and zone five respectively. At cathodal end three zones were identified which were represented by 2, 5 and 4 bands respectively.

6.3.2. Interspecific diversity for different isozymes

Interspecific diversity for Esterase : Out of total 18 bands distributed over 25 species, Band 3 was represented in maximum 13 species followed with Band 13 in 12 species. Six bands were considered as species specific viz. Band 1 in *T. purpureum*, Band 2 in *T. medium*, Band 9 in *T. repens*, Band 15 in *T. incarnatum*, Band 17 in *T. resupinatum* and band 18 in *T. angustifolium*. The highest number of 8 bands were present in *T. resupinatum*. Three species viz. *T. lappaceum*, *T. diffusum* and *T. constantinopolitanum* were represented by a single band.

Interspecific diversity for Super-oxide-dismutase isozyme : Out of total 8 bands identified for the genus, Band 4 was found to be most common and was represented in 22 species except *T. diffusum*, *T. retusum* and *T. spumosum*. The second highest frequency was that of band 7 which was present in 21 species and absent in *T. hirtum*, *T. echinatum*, *T. campestre* and *T. argutum*. Band 2 was present in 18 species. Band 5 was represented in only 7 species while band 1 was present in three species i.e. *T. purpureum*, *T. angustifolium* and *T. medium*. Band 3 was uniquely present in two out of three accessions of *T. hirtum*. Band 6 and 8 were species specific and present only in *T. echinatum* and *T. hirtum* respectively.

Interspecific diversity for Glutamate-oxalo-acetate transaminase (GOT) : Total 10 bands distributed over 25 species were identified. One to five bands were represented in various species. Of these, Band 7 was most common and present in 21 species and absent in *T. repens*, *T. pratense*, *T. nigrescens* and *T. lappaceum*.

Band 6 present in 14 species was second most common band. Band 4 and 5 were present in only four species each. Band 4 was present in *T. subterraneum*, *T. alexandrinum*, *T. hirtum* and *T. diffusum*. Band 1 was present in only two species i.e. *T. campestre* and *T. incarnatum*. Band 5 was represented in four species namely *T. hirtum*, *T. campestre*, *T. diffusum* and *T. tembense*. The maximum five bands were present in four species i.e. *T. hirtum*, *T. alexandrinum*, *T. spumosum* and *T. tembense*. The species with four bands were *T. subterraneum*, *T. campestre*, *T. incarnatum*, *T. diffusum* and *T. alpestre*. The species represented with three bands only were *T. nigrescens*, *T. medium*, *T. constantinopolitanum*, *T. retusum*, *T. argutum* and *T. apertum*. *T. lappaceum* showed presence of only band i.e. Band 6.

Interspecific diversity for Acid Phosphatase (ACP) : Based on relative mobility of bands, a total of eight bands were found to be distributed throughout genus. The slowest band no.1 was represented in all the 25 species studied, hence, was identified as genus specific band. The second highest frequency was that of Band 6 which was present in 15 species. Band 3 was present in four species (*T. subterraneum*, *T. hirtum*, *T. incarnatum* and *T. spumosum*.) Band 2 was present in three species viz. *T. resupinatum*, *T. argutum* and *T. tembense*. Band 4 was present in *T. subterraneum*, *T. medium* and *T. alexandrinum*. Band 7 was present only in two species i.e. *T. incarnatum* and *T. diffusum*. Band 5 and 8 were found to be species specific bands. Band 5 was specific to *T. resupinatum* and Band 8 specific to *T. incarnatum*. A total of 14 species possessed only two bands whereas five species showed presence of one band only. Three species were having three bands and another three possessed four bands.

Interspecific diversity for Peroxidase enzyme : Eleven bands each at anodal and cathodal ends were identified based on their relative mobility. These were distributed in different species in various combinations. Band A11 was invariably present in all 25 species, this was identified as genus specific. The second highest frequency was that of band A7 (present in 16 species) followed with band A5 which was present in 10 species. Band A1 was present only in two species i.e. *T. repens* and *T. glomeratum* while band A3 in three species i.e. *T. subterraneum*, *T. resupinatum* and *T. retusum*. Band A8 was also present in only 3 species namely *T. pratense*, *T. subterraneum* and *T. incarnatum*. Some bands were found species

specific such as band A2 in *T. resupinatum*, band A4 in *T. hirtum*, band A9 in *T. pratense*, band A10 in *T. campestre* and band A6 in *T. diffusum*. The highest number of four anodal bands were present in *T. repens*, *T. subterraneum*, *T. hirtum* and *T. resupinatum*. Most of the species possessed two anodal bands. *T. spumosum* was represented with single anodal band.

Among cathodal peroxidase bands the highest frequency was that of band C4 which was present in 16 out of 25 species. Next highest frequency was of band C7 which was present in 15 species followed by band C2 and C9 in 14 species each. Some bands were found in single species and identified as species specific bands such as band C1 in *T. hybridum* band C6 in *T. purpureum*, band C8 and C10 in *T. repens*. The maximum number of bands in any species was six in *T. repens* and *T. resupinatum*. Ten species were represented with 3 bands, six species with 2 bands, five species with 4 bands and *T. diffusum* with single band.

6.3.3. Intraspecies similarity among different species of *Trifolium*

Intra-species similarity was calculated only for the species which were represented by more than five accessions.

The dendrogram based on five enzyme system of nine accessions of *T. repens* showed the presence of three clusters. In cluster No 1, EC 401704 was 92% similar with EC 401707. Cluster no. 1 showed 75% similarity with cluster number 2 comprising of five accessions. In cluster no. 2, EC 400986 was 100% similar to EC 400985 and showed 87% similarity with EC 401706. Eighty percent similarity was observed between EC 400984 with other three accessions (EC 400986, EC 400985 and EC 401706). In cluster No 3, 93% similarity was observed between EC 401705 and EC 401708 and cluster 3 showed 72% similarity with other clusters.

Twelve accessions of *T. resupinatum* showed the presence of five clusters as per dendrogram obtained on the basis of five enzyme system. In cluster no. 1, SH 98-86, SH 98-73 and SH 98-36 showed 84% within group similarity. Cluster number 2 was 72% similar to cluster no. 1. JHS-3 made separate cluster i.e. cluster no. 3 which showed 67% similarity with cluster no. 4 and 5. In cluster 4, SH-99-25 was 93% similar with SH-99-29. In cluster 5, SH-99-33 showed 97% similarity

with SH-99-69 whereas SH-99-23 was 95% similar with these two. SH-99-32 showed 91% similarity with group of SH-99-69, SH-99-33 and SH-99-23 accessions. SH-99-26 showed 90% similarity with rest of the accessions of cluster no. 5.

Eight accessions of *T. pratense* were grouped in 2 clusters on the basis of dendrogram obtained after analyses of five enzyme system. 100% within group similarity was found in both the clusters. Cluster number 1 showed 96.6% similarity with cluster number 2.

A total ^{of} 13 clusters were observed after cluster analysis ^{was} done on the basis of five enzyme system of 65 germplasm lines of *T. alexandrinum*. Cluster number 1 comprising of single accession i.e. EC 329299 showed 79% similarity with rest of the clusters. All remaining clusters showed more than 90% similarity among themselves. Cluster number 2 comprised of seven lines, out of which six lines showed 100% similarity and together they were 97.7% similar with IL 40010. A total of 13 lines were observed in cluster number 3, which showed 97% within group similarity. This cluster showed 96.6% similarity with cluster no. 2.

Cluster 4 showed 95% similarity with combination of cluster no. 2 and 3. In cluster number 5, two similar lines showed 97% similarity with other 5 lines which were 100 % similar. This cluster was observed to be 95.2% similar with group of cluster no. 2,3 and 4. Cluster number 6 showed 95% similarity between two lines and was 93% similar with group of cluster no. 7, 8 and 9. In cluster number 7, BL 122 was 100% similar to Raj 7/49-50 and these two accessions showed 98% similarity with JHB 146. In cluster number 8, five lines, which were 100% similar, showed 97% similarity with group of two lines which were also 100% similar. This cluster showed 96.6% similarity with cluster no. 9, comprising of 8 lines of 100% similarity.

The group comprising of cluster no. 2 to 9 showed 89.8 % similarity with another group of cluster no. 10 to 13. Cluster number 10, in which 4 lines were included showed 95% within group similarity. This cluster showed 92.7% similarity with cluster 11, 12 and 13. In cluster number 11, four lines were clustered and JHB P17-1 showed 97% similarity with group of 3 lines which were 100% similar.

Cluster number 12 in which 3 lines were included, showed 100% intra cluster similarity. This cluster showed 96% similarity with cluster no. 11. In cluster number 13, three lines were included. JHB P/T-34 showed 100% similarity with Raj 7/13-14 and 97.3% similarity with JHB 94-R-13.

In *T. alexandrinum*, 65 lines were analysed which formed 3 major groups which were again subdivided into 13 clusters. Group I comprising of single accession (EC 329299) was different ecotype ('Saidi') and has wide difference for morphological features and regeneration potential. Group II comprised of 50 lines which were grouped into cluster 2 to 9. This group showed 93% within group similarity. Many of the accessions were 100% similar indicating thereby presence of duplicates. Group III comprised of 14 accessions grouped into clusters 10 to 13. This group showed 92% within group similarity^{and} was 89.8% similar with group II. The 'Saidi' type accession showed 79.2% similarity with 'Mescavi' type accessions represented by 64 accessions grouped in cluster 2 to 13.

6.3.4. Interspecies similarity among different *Trifolium* species

Dendrogram obtained on the basis of five enzyme system of 25 *Trifolium* species showed the presence of following 7 clusters.

Cluster no.	Species
1	<i>T. repens</i>
2	<i>T. pratense</i> , <i>T. cherleri</i> , <i>T. spumosum</i> , <i>T. subterraneum</i> , <i>T. resupinatum</i> , <i>T. alexandrinum</i>
3	<i>T. nigrescens</i> , <i>T. glomeratum</i> , <i>T. apertum</i> , <i>T. alpestre</i> , <i>T. hybridum</i> , <i>T. echinatum</i>
4	<i>T. purpureum</i> , <i>T. angustifolium</i> , <i>T. medium</i> , <i>T. lappaceum</i> , <i>T. constantinopolitanum</i> , <i>T. tembense</i>
5	<i>T. retusum</i>
6	<i>T. hirtum</i> , <i>T. campestre</i> , <i>T. incarnatum</i> , and <i>T. argutum</i> .
7	<i>T. diffusum</i> .

T. repens, *T. retusum* and *T. diffusum* each made clusters of single species i.e. clusters number 1, 5 and 7 respectively. *T. repens* forming independent cluster showed 46% similarity with cluster no. 2 to 6. In cluster number 2, *T. spumosum* and *T. cherleri* showed highest affinity (78.8%) between themselves and these two species together showed 64.7% similarity with *T. pratense*. In this cluster *T.*

alexandrinum was 72.4% similar to *T. resupinatum*. Moderate similarity of 62.5% was present between *T. subterraneum* and the group of *T. alexandrinum* and *T. resupinatum*.

In cluster number 3, six species were clustered. Out of these *T. hybridum* and *T. alpestre* made a small sub-cluster of 77.4% similarity and 61.4% similarity with *T. echinatum*. Another sub-cluster of *T. apertum*, *T. glomeratum* and *T. nigrescens* was found which showed 63.7% similarity among themselves. The group of three species i.e. *T. hybridum*, *T. alpestre*, and *T. echinatum* showed 58.6% similarity with group of *T. apertum*, *T. glomeratum* and *T. nigrescens*. Cluster number 2 and 3 were observed to be 56.4 % similar.

In cluster 4, *T. angustifolium* showed 68.6% similarity with *T. purpureum* whereas *T. constantinopolitanum* was found 72% similar to *T. lappaceum*. 64 % similarity was observed between *T. tembense* and *T. constantinopolitanum*. 57.9% similarity was observed between *T. medium* and the other group of three species i.e. *T. constantinopolitanum*, *T. lappaceum* and *T. tembense*. Cluster no. 5 comprising of *T. retusum* showed 49.9% similarity with combination of cluster no. 2, 3 and 4.

Cluster number 6 comprised of 4 species and made two sub clusters. *T. campestre* was 55% similar to *T. hirtum* and *T. argutum* showed 51.3% similarity with *T. incarnatum*. These two sub clusters showed 48% similarity. Cluster no. 6 showed 47.1% similarity with group of clusters 2 to 5. Similarly, this group of clusters 2 to 6 was 46.1% similar with cluster no. 1. *T. diffusum* made separate cluster (No. 7) and showed only 40.5% similarity with rest of the clusters.

The dendrogram obtained on the basis of five enzyme system of the 134 accessions of 25 *Trifolium* species showed the presence of 19 clusters.

Cluster no. 1 in which nine accessions of *T. repens* were included showed 48.9% similarity with group of cluster no. 2 to 16. In cluster no. 2, eight accessions of *T. pratense* were included which showed 97% similarity within them. Cluster No 2 showed 64.4% similarity with cluster no.3.

In cluster number 3, single accessions each of two species i.e. *T. cherleri* and *T. spumosum* were present. *T. spumosum* showed 78.8% similarity with *T. cherleri*. In cluster no. 4, all five accessions of *T. subterraneum* were present. EC 402167 showed 77% similarity with EC 401717 and both showed 91% similarity with EC 401718. 100% similarity was observed between IG 96-112 and IG 96-113. *T. constantinopolitanum* (EC 401713) made separate cluster i.e. cluster no. 5 and showed 75.8% similarity with cluster no. 6 comprising of accessions of *T. alexandrinum*.

All 65 accessions of *T. alexandrinum* were present in cluster No 6. Except EC 329299, the rest 64 accessions showed >90% similarity among themselves, whereas EC 329299 (a Saidi type) was 79% similar to rest of accessions. Cluster 5 and 6 showed 70% similarity with clusters no.7 (comprising of single accession of *T. tembense*).

T. medium (EC 425045) was present in cluster number 8 and showed 59.8% similarity with cluster no.9. In cluster no. 9, *T. apertum* (EC 401712) showed 73% similarity with two accessions of *T. glomeratum* which were 100% similar. In cluster no. 10, two accessions of *T. echinatum* were present which showed 74% similarity.

Two accessions of *T. alpestre* made separate cluster (cluster no. 11) which were 92% similar and this cluster was 70.2% similar with cluster no. 12 of *T. hybridum* (comprising of five accessions). In cluster no. 13, two accessions each of two species i.e. *T. purpureum* and *T. angustifolium* were present in two small sub clusters. Eighty seven percent similarity was observed between two accessions of *T. purpureum* and the two accessions of *T. angustifolium*. Sixty three percent similarity was observed between *T. purpureum* and *T. angustifolium*. This cluster showed 54.1% similarity with group of clusters no. 2 to 12. Twelve accessions of *T. resupinatum* made separate cluster and are represented in cluster no. 14. This cluster showed 56.5% similarity with cluster no. 15 of *T. retusum* (EC 402150). In cluster no. 16, three accessions of *T. hirtum* were included. Eighty six percent similarity was observed between EC 425038 and EC 425037 whereas EC 425039 showed 78% similarity with both these accessions. Cluster no. 16 showed 48.3% similarity

with group of cluster no. 2 to 15. Cluster no. 1 of *T. repens* showed 48.9% similarity with various species grouped in clusters 2 to 16.

Three accessions of *T. nigrescens* were present in cluster number 17. Ninety six percent similarity was observed between EC 425048 and EC 425049 and these together showed 63% similarity with EC 425047. This cluster showed 44.8% similarity with cluster no. 1 to 16. Single accessions each of *T. lappaceum* and *T. diffusum* were included in cluster No 18. and 57% similarity was observed between these two species. Cluster No. 18 showed 44.6% similarity with cluster no.1 to 17. In cluster no. 19, three sub clusters were observed. Two accessions of *T. campestre* were observed to be 86.9% similar. IG 96-111 and EC 402164 of *T. incarnatum* showed 95.5% similarity. *T. argutum* was 54.1% similar with *T. incarnatum*. This cluster showed 44.6% similarity with remaining cluster i.e. 1 to 18.

Wide interspecific genetic diversity was observed among *Trifolium* species. *T. diffusum* was most distantly placed species out of 25 studied and showed only 40.5% similarity with other 24 species. *T. repens* also fell at the other end of dendrogram and was only 46.1% similar with other 23 species. Moderate affinity (> 70 %) was observed between *T. cherleri* and *T. spumosum* (78.8%), *T. alpestre* and *T. hybridum* (77.4%), *T. alexandrinum* and *T. resupinatum* (72.4%), *T. glomeratum* and *T. apertum* (72.7%) and that between *T. lappaceum* and *T. constantinopolitanum* (72%).

It is evident from the isozymic pattern from different enzymes that the 65 accessions of *T. alexandrinum* possessed very little variability. In most of the cases the similarity was observed to be 95-100% except the accession number EC 329299, (a 'Saidi' type) line which was 79% similar to rest of accessions. There was no marked difference for banding pattern among the *T. alexandrinum* lines representing various climatic conditions, except for one or two bands of peroxidase, one band of each of SOD and ACP and two bands of GOT. *T. alexandrinum* lines representing various exotic lines, some collection each from Rajasthan, Punjab, Haryana, tetraploid lines, pentafoolate and red flowered plants of *T. alexandrinum* possessed 95-100% similarity, although, the red flowered and pentafoolate plants clustered at the end of the dendrogram. These berseem accessions were 75% similar with *T.*

constantinopolitanum at one end and *T. alexandrinum* together with *T. constantinopolitanum* formed one cluster which was 70% similar with *T. tembense*. Thus, these two species i.e. *T. tembense* and *T. constantinopolitanum* seemed to be closest to *T. alexandrinum*. The other few species which showed close affinity with *T. alexandrinum* were *T. medium*, *T. glomeratum*, *T. apertum* and *T. echinatum* in the order of similarity. Species like *T. lappaceum*, *T. diffusum*, *T. campestre*, *T. incarnatum* and *T. argutum* were clustered at the end of the dendrogram and showed only 44.8% similarity with other *Trifolium* species. If we look in to the origin and domestication of these species all these five species have domesticated in Europe and USA. Except for *T. incarnatum* from this group rest of the species showed less number of isozyme bands represented in them. Some of widely cultivated species in India like *T. alexandrinum* and *T. resupinatum* possessed 28 and 27 bands respectively out of total 66 bands identified but these five species showed presence of 11 to 17 bands only. The two widely cultivated and domesticated (for about a century) species i.e. 'Berseem' and 'Shaftal' in India show higher number of bands than European and Western species.

Based on pooled zymogram pattern *T. alexandrinum* showed maximum affinity with *T. resupinatum* (72% similarity) followed with *T. apertum* (71%). *T. purpureum* and *T. subterraneum* also showed 69% and 68% similarity with *T. alexandrinum*, whereas *T. campestre* and *T. lappaceum* were most distantly related with *T. alexandrinum*.

T. constantinopolitanum showed closer affinity with *T. lappaceum* (72%), *T. tembense* (71%), *T. apertum* (71%) and *T. echinatum* (71%) as compared to 63% similarity with *T. alexandrinum*, although on the basis of crossability study using embryo rescue method, moderate genetic affinity between *T. alexandrinum* and *T. constantinopolitanum* was observed. The higher degree of crossability of *T. alexandrinum* with *T. apertum* as well as banding pattern similarity of *T. apertum* with *T. constantinopolitanum* lead^s to think that *T. apertum* might be a bridge species of the cross between *T. alexandrinum* x *T. constantinopolitanum*. The semi erect habit of *T. apertum* also looks to be intermediate nature of plant type of erect *T. alexandrinum* and prostrate *T. constantinopolitanum*. The maximum affinity (79%) between any two species was observed between *T. spumosum* with *T. cherleri*. From

the similarity index table it is clear that the species closer to (more than 60% similar) *T. alexandrinum* are *T. resupinatum* (72%), *T. apertum* (71%), *T. purpureum* (69%), *T. subterraneum* (68%), *T. nigrescens* (65%), *T. cherleri* (64%), *T. argutum* (64%) and *T. spumosum* (63%). The species which showed close affinity with the two closest species of *T. alexandrinum* i.e. *T. resupinatum* and *T. apertum* were also identified based on similarity index. The closest species to *T. resupinatum* was *T. alexandrinum* itself followed with *T. medium* (67% similarity) whereas the closest species to *T. apertum* was *T. tembense* (73%) followed with *T. alexandrinum*, *T. alpestre* and *T. constantinopolitanum* (71% similar). Thus, there is possibility of *T. alexandrinum* and *T. resupinatum* commonly evolving from *T. medium* at one hand and *T. alexandrinum* alone evolving from *T. constantinopolitanum* through *T. apertum*.

6.3.5. Estimation of intra species variability

A total of 46 types of zymograms for esterase isozyme pattern were observed in 25 different species of *Trifolium* and this difference was due to the presence or absence of any one or more than one of all the 18 bands. In case of *T. resupinatum* and *T. hybridum* the maximum number 8-9 bands contributed towards the variability whereas in many other cases only 1-2 bands were responsible for intra species zymogram variation. The estimates of variability revealed maximum variation for zymograms pattern in *T. resupinatum* (4.24) followed with 3.01 in *T. nigrescens*. Fourteen species showed no interspecies variation and estimation of cumulative estimate of variability revealed high degree of variation for types of zymogram represented in different *Trifolium* species, the variability estimate was recorded as 4.38.

SOD isozyme banding pattern observed in different species of *Trifolium* revealed that 2-4 bands out of total 8, contributed towards variability among six species only whereas 19 species showed no intra-species variation for SOD banding pattern. Estimate of variability also ranged from 0.458 to 2.667 only. The total estimate of variability across species was also quite less (1.082) as compared to estimate of variability for esterase.

In case of GOT isozyme banding pattern only 2 species showed intra-species variation for zymogram pattern but a total of 26 types of zymograms were noticed among different species accounting for 2.306 estimate of variability. All the 10 bands contributed for intra-species variation

Out of total 8 isozyme bands for ACP observed among different *Trifolium* species Band number 1 was common to all. The remaining 7 bands contributed towards an estimate of variability of 1.428 in the genus. Sixteen different types of ACP zymograms were noticed. The intraspecies zymogram variation was observed only in 6 species and 1-3 bands were found different among different accessions of same species. Highest variation for intraspecies zymogram pattern was observed in *T. resupinatum* (4.526).

Zymogram pattern based on 11 anodal and 11 cathodal bands of Peroxidase enzyme showed high degree of variation among different species of the genus. Overall estimate of variation was to the tune of 4.833 and 51 types of zymogram were observed among different species. Out of 22 bands observed one band (A11) was common to all and the rest 21 bands accounted for different zymogram patterns. The intraspecies variation for types of zymogram was observed in 9 species. One to nine bands contributed towards intra-species variation for zymogram pattern. The estimate of variation ranged from 0.938- 3.359. The highest variation was recorded in *T. repens* i.e. 3.359.

T. repens, *T. subterraneum*, *T. resupinatum*, *T. echinatum*, *T. hirtum*, *T. campestre* and *T. incarnatum* had high values for variability estimates whereas *T. alexandrinum* showed very little estimate of variation. Except for esterase isozyme, *T. alexandrinum* was noticed for presence of more than one type of zymogram for different enzyme.

Each species possessed its peculiar zymogram for esterase isozyme except one zymogram which was common to *T. constantinopolitanum* and one accession of *T. hybridum*. The presence of number of bands also varied from 1 to 6.

Generally, the interspecies variability for esterase zymogram was larger than the intra species variation and almost all the species had their specific zymogram.

Presence of only one type of zymogram among 65 accessions of *T. alexandrinum* shows that the species has no variation for esterase isozyme. The bands of *T. alexandrinum* were found scattered in different accession of *T. resupinatum*. It also showed 2 bands common with *T. apertum*. In all, one or more bands of *T. alexandrinum* were found common with 20 species under study. The four species i.e. *T. argutum*, *T. diffusum*, *T. incarnatum* and *T. constantinopolitanum* possessed no common band with *T. alexandrinum*.

6.4. Protein analysis

The electrophoretic pattern for native leaf protein of 12 species of *Trifolium* was carried out on PAGE. In all, 28 electrophoretic bands distributed throughout the genus were recorded. None of the species showed identical banding pattern. Out of four samples of *T. alexandrinum*, three samples belonged to self progeny of accession number JHB 99-32 and one of JHB 99-25. The two samples of selfed progeny of JHB 99-32 and one of JHB 99-25 showed identical banding pattern i.e. presence of Band 1, 2, 3, 5, 6, 16, 18, 19, 22, 23, 25, and 27 whereas one plant in the self progeny of JHB 99-32 showed presence of Band number 2, 3, 16, 17, 19, 22, 23, 25, and 27. The maximum number of bands present in any species was 13 in *T. purpureum* (band 1, 2, 3, 5, 6, 7, 8, 11, 16, 17, 18, 19 and 27) and *T. vesiculosum* (band 1, 2, 3, 11, 12, 14, 15, 16, 17, 20, 22, 25 and 27) and the minimum 4 bands were found in *T. echinatum* (16, 19, 22, 28) followed with 6 bands in *T. pratense*. Band number 3 was most commonly represented and was found in 11 out of 12 species studies. Band 4 and 9 in *T. hybridum*, Band 10 in *T. angustifolium*, Band 15 in *T. vesiculosum*, Band 23 in *T. alexandrinum* and Band 24 and 26 in *T. resupinatum* were found to be species specific.

On the basis of similarity index, *T. alexandrinum* lines showed maximum affinity with *T. apertum* (70-87%) followed with *T. purpureum* (72%) and *T. vesiculosum* (56-62%). Fifty four per cent similarity of *T. alexandrinum* was also observed with *T. hirtum*. Accessions of *T. alexandrinum* showed maximum distance from *T. pratense* (26 to 33% similarity) followed with *T. hybridum* (30% similarity) and 37-46% with *T. echinatum*. Thus, *T. alexandrinum* can be said to have closest affinity with *T. apertum* followed with *T. purpureum*. It is also to note

that *T. alexandrinum* has 11 bands (out of total 12) common with *T. apertum* and 9 bands common with *T. purpureum*, although *T. purpureum* and *T. apertum* both had some additional bands and some being absent. The next close species to this group of three species (*T. alexandrinum*, *T. apertum* and *T. purpureum*) was *T. vesiculosum* showing 57% similarity with this cluster. This group of 4 species together was 52% similar with another group of three species comprising of *T. resupinatum*, *T. repens* and *T. lappaceum*. *T. angustifolium*, *T. pratense*, *T. echinatum*, *T. hybridum* and *T. hirtum* formed small group of 1 to 2 species and were placed quite away from the cluster of *T. alexandrinum*.

The genotypes of berseem represented by two major groups i.e. diploid and tetraploid were analysed for crude protein content. Both the groups showed genotypic variations. Crude protein (CP) content varied from 18.88 to 21.32% in diploid and from 19.81 to 22.52 in tetraploid lines. Almost all the cultivars showed better crude protein content than that of the national check 'Wardan'. A few lines, viz JHB 94- -R-25, JHTB 9-90-N1 and JHTB 1-90-A1 showed 2 to 3% higher CP% compared to Wardan.

6.5. Interspecific hybridization

The major genes for wide scale adaptability to different biotic and abiotic stress are reported to be widely distributed in several wild species of *Trifolium* which could be incorporated into the present day cultivars provided that the problem of interspecific crossability is overcome.

The compatibility study was conducted by attempting interspecific crosses in following combinations:

T. alexandrinum x *T. apertum*, *T. alexandrinum* x *T. constantinopolitanum*,
T. alexandrinum x *T. echinatum*, *T. alexandrinum* x *T. repens*, *T. alexandrinum* x *T. pratense*, *T. alexandrinum* x *T. resupinatum*, *T. alexandrinum* x *T. incarnatum*, *T. alexandrinum* x *T. vesiculosum*, *T. alexandrinum* x *T. hybridum*, *T. alexandrinum* x *T. purpureum*.

The various parameters observed were withering of floral parts, development of ovary/ ovule, successful recovery of ovule/embryo, germination of embryos in aseptic nutrient media *in vitro*.

The study indicated that there was no response of pollinating *T. alexandrinum* with *T. incarnatum* and negligible response with *T. vesiculosum* and *T. repens*. Thus, these species have no affinity with *T. alexandrinum*. Successful embryo germination in the *T. alexandrinum* crossed with *T. apertum*, *T. constantinopolitanum*, *T. echinatum* and *T. resupinatum* shows that these four species have closer affinity with *T. alexandrinum* than other species. It has been observed that in the normal course there are two ovules in *T. alexandrinum* and after fertilization one ovule gets degenerated and the other develops. The similar phenomenon was observed in *T. alexandrinum* x *T. apertum* cross wherein development of only one ovule was observed in the withered flowers, whereas both the ovules remained healthy for a very long period where no fertilization occurred. In other crosses involving *T. constantinopolitanum*, *T. echinatum*, *T. pratense* *T. resupinatum* 0.7, 0.3, 0.11 and 0.22 ovule per ovary were observed respectively. If this is considered as criteria for measuring affinity of species *T. constantinopolitanum* and *T. apertum* seemed to be the closest species to *T. alexandrinum* followed with *T. resupinatum* and *T. echinatum*.

6.6. Conclusion

Most of the earlier studies about phylogeny and classification of the genus were based on morphological studies and geographical distribution. Temperate species were also compared phylogenetically on the basis of interspecific hybridization and cytology. Reports based on molecular and biochemical parameters were lacking on the tropical species.

Thus, the present study on genetic similarity at inter and intra specific level among different accessions belonging to 26 species of the genus is an attempt to get a better insight into the relatedness of different species with particular reference to *T. alexandrinum*.

The study confirmed the earlier observations that the genetic base of berseem is quite narrow. Out of 65 accessions studied by isozymic method more than 95% similarity was observed among 64 accessions. The only accession (EC 329299), a 'Saidi' ecotype was somewhat different.

Earlier reports indicated similarity among *T. alexandrinum*, *T. apertum*, *T. constantinopolitanum*. Our study has also shown high genetic similarity based on isozyme analysis, supported by cytological and crossability studies that these three species as well as *T. echinatum* and *T. resupinatum* are closely related. Successful embryo germination after interspecific hybridization has been obtained in these crosses.

T. alexandrinum was found to be closer to another cultivated clover (*T. resupinatum*) in sub-tropical part of India. Isozymic study show moderate similarity between these two species (72%). Cytological and crossability study also confirm this similarity and *in vitro* embryo germination could successfully be obtained in this cross.

Isozymic study was found to be more reliable than the morphological observations. Clustering of 134 accessions belonging to 25 different species showed no overlapping of accessions. All the accessions of any particular species were placed together in the dendrogram, thereby confirming the high efficacy of this analysis.

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